Genome-scale identification of MLO domain-containing genes in soybean (*Glycine max* L. Merr.)

Qi Shen^{1,2†}, Jinming Zhao^{1†}, Caifu Du², Yang Xiang^{1,2*},
Jinxuan Cao³ and Xinrong Qin²

¹National Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University,
Nanjing 210095, P.R.China

²Guizhou Rapeseed Institute, Guizhou Academy of Agricultural Sciences, Guiyang 550008, P.R.China

³College of Life Science and Biotechnology, Ningbo University, Ningbo 315211, P.R.China.

(Received 9 June 2011, accepted 27 February 2012)

In plants, powdery-mildew-resistance locus o (*Mlo*) genes encode proteins that are calmodulin-binding proteins involved in a variety of cellular processes. However, systematic characterization of this gene family in soybean (*Glycine max* L. Merr.) has not been yet reported. In this study, we identified MLO domain-contained members in soybean and examined their expression under phytohormone treatment and abiotic stress conditions. A total of 20 soybean *Mlo* genes were identified (*GmMlo1-20*), which are distributed on 13 chromosomes, and display diverse exon-intron structures. Phylogenetic analysis indicated that the *Mlo* family can be classified into four subfamilies. Sequence comparison was used to reveal the conserved calmodulin-binding domain (CaMBD) in GmMLO proteins. The expression of Gm*Mlo* genes was influenced by various phytohormone treatments and abiotic stresses, suggesting that these *Mlo* genes have various roles in the response of soybean to environmental stimuli. Promoter sequence analysis revealed an overabundance of stress and/or phytohormone-related *cis*-elements in Gm*Mlo* genes. These data provide important clues for elucidating the functions of genes of the *Mlo* gene family.

Key words: soybean; MLO domain; gene family; abiotic stress; phytohormone

INTRODUCTION

In plant cells, the calcium ion (Ca²⁺) is a ubiquitous intracellular second messenger involved in numerous signaling pathways. It also affects diverse cellular and developmental processes, including modulation of apoptosis, stress responses, organogenesis and transcriptional activity (Yang and Poovaiah, 2003). Calcium signaling is decoded and transduced by an array of Ca²⁺-binding proteins, giving rise to a cascade of downstream calcium-dependent events (Rudd and Franklin-Tong, 2001; Sanders et al., 2002). Some genes have been identified that are involved in the early stage of calcium signaling transduction; for example, calmodulin (*CaM*), pathogen-induced CaM-binding protein (*PICBP*), calcineurin B-like (*CBL*), powdery mildew resistance locus o (*Mlo*), *Arabidopsis thaliana* stress responsive genes (*AtSR*), calcium-dependent protein kinases (*CDPKs*) and CaM-binding protein 60 g (*CBP60g*) (Büschges et al., 1997; Zielinski,

1998; Reddy, 2001; Hrabak et al., 2003; Du et al., 2009; Wang et al., 2009).

Mlo genes are a particular class of plant-specific CaM-binding protein genes (Wolter et al., 1993; Kim et al., 2002a, 2002b). The *Mlo* gene was first isolated in barley (Büschges et al., 1997) and, since then, genes encoding MLOs have been identified in several plant species, including 15 *Mlos* in *Arabidopsis thaliana*, seven in wheat and 12 in rice (Devoto et al., 2003; Liu and Zhu, 2008; Konishi et al., 2010). Accumulating data indicate that MLO proteins have a characteristic structure comprising seven transmembrane (TM) domains and a C-terminal CaM-binding domain (CaMBD) (Büschges et al., 1997; Devoto et al., 1999; Kim et al., 2002a, 2002b). Binding of CaM to MLO mediates a Ca²⁺-dependent pathogen defence reaction (Kim et al., 2002a, 2002b). Thus, the *Mlo* gene is known as an early Ca²⁺-responsive gene.

Recently, *Mlos* have attracted increasing attention because of their association with the stress responses in plants. In barley (*Hordeum vulgare*), homozygous recessive mutant alleles of the *Mlo* gene can confer durable and broad-spectrum pathogen resistance to the biotrophic

Edited by Jaroslav Dolezel

* Corresponding author. E-mail: xiangyangcell@yahoo.com

† These authors contributed equally to this work

powdery mildew fungus (Jørgensen, 1992; Piffanelli et al., 2002, 2004). Loss of *Arabidopsis MLO2* plant produced resistance against multiple powdery mildew species (Consonni et al., 2006, 2010). The same defense mechanism was found in tomato *MLO1* (Bai et al., 2008). Another main role of *Mlo* in plants is in the regulation of cell death; for example, mutant *mlo* plants exhibit spontaneous mesophyll cell death associated with partly accelerated leaf senescence (Wolter et al., 1993; Piffanelli et al., 2002; Stein and Somerville, 2002; Consonni et al., 2010). An increasing number reports suggest that *Mlos* also have additional functions in response to abiotic stimuli. For example, there are some evidences to suggest that *Mlo* transcript abundance increased in response to wounding, paraquat treatment, a wheat powdery mildew-derived carbohydrate elicitor, salt stress and mannitol treatment (Piffanelli et al., 2002; Feechan et al., 2008; Konishi et al., 2010). However, despite the apparent association of *MLOs* with plant abiotic stresses, their roles in plants remain elusive.

Soybean (*Glycine max* L. Merr.) is one of the most economically oil crops worldwide. Based on a comparison with *MLOs* in other plant species, those in soybean should also be encoded by the same multi-gene family. However, *Mlo* genes have not yet been identified in soybean. Sequencing of the soybean genome provides an opportunity to identify previously uncharacterized genes. In this study, a bioinformatics approach was used to identify all members of the *Mlo* gene family in soybean. Their genomic organization, Ca^{2+} -binding motif location, phylogenesis, *cis*-elements in promoter regions and the expression profiles under various abiotic and hormone stresses were analyzed in detail. The results presented here provide important information for future function studies of *Mlo* genes.

MATERIALS AND METHODS

Identification of *Mlo* members in soybean The HMMER (version 2.3.2) program was used to search all *MLO* domain-containing protein genes in the soybean genome databases (<http://www.phytozome.net/soybean.php>, phytozome, Release v4.0) with the HMM (Hidden Markov Model) profile of the *MLO* domain (PF03094) (<http://pfam.sanger.ac.uk/Software/Pfam>; Finn et al., 2006) and with an E-value < 1.0e-200 and more than 450 amino acid residues as the cutoff. The predicted *MLO* sequences were then compared against the conserved *MLO* domain in the Pfam and SMART (<http://smart.embl-heidelberg.de>; Letunic et al., 2004) databases.

Database search and sequence analysis Expressed sequence tags (ESTs) were subjected to a BLAST search against the identified *Mlo* sequences to determine genuine EST hits. The exons-introns structures in the differ-

ent genes were determined by comparing of the cDNAs with their corresponding genomic DNA sequences. Open reading frame (ORF) analyses were performed with the ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The exons and introns were determined by using the Gene Structure Display Server (GSDS) tool (<http://gsds.cbi.pku.edu.cn/>). TM segments were predicted by using the TMHMM2 tool (<http://www.cbs.dtu.dk/services/TMHMM/>; Krogh et al., 2001). Motifs were identified statistically using online MEME (<http://meme.sdsc.edu>). CaM-binding motif analysis was identified according to Kim et al. (2002b). The chromosomal positions of *GmMlo* genes were also retrieved from phytozome soybean genome databases (<http://www.phytozome.net/soybean.php>, phytozome). Ten proteins-encoding genes from regions flanking of each *GmMlo* gene were retrieved from phytozome soybean genome databases to identify segmental replication events of *GmMlos*.

Protein sequence alignments and phylogenetic analysis Multiple alignments of protein sequences were performed using Clustal W (Accelrys Inc., USA). A phylogenetic tree was constructed using neighbour-joining (NJ) algorithms. Bootstrapping was performed 1000 times to obtain support values for each branch.

***cis*-elements identification** To analyze putative *cis*-acting regulatory DNA elements (i.e. *cis*-elements) in the promoters of *GmMlo* genes, online PLACE 26.0 (<http://www.dna.affrc.go.jp/PLACE/index.html>; Lescot et al., 2002) was used to analyze a 2,000-bp sequence upstream of the full-length cDNAs or predicted CDS extracted from the genomic sequences.

Plant materials, abiotic and phytohormone treatments The plant materials used in this study included the soybean cultivars 'Williams'. Plants were grown in a controlled environment chamber (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 14 h light/10 h dark per day at $22 \pm 2^\circ\text{C}$).

In the abiotic treatment, 14-day-old seedlings were treated at either 40°C or 4°C , or with either 5% polyethylene glycol (PEG) or 200 mM NaCl. In the 40°C treatment, the leaves were freeze-stored 1 h after the heat shock treatment. In the 4°C treatment, the leaves were collected 6 h after treatment. For the PEG and NaCl treatments, the leaves were then frozen in liquid nitrogen after 24 h of treatment. Untreated seedlings were used as controls.

To obtain phytohormones-treated leaf tissues, 14-day-old seedling leaves were sprayed with either 10 mM abscisic acid (ABA), 1 mM 6-benzyladenine (6-BA), 10 mM gibberellic acid (GA_3) or 1 mM indole acetic acid (IAA) (all plus 0.1% (v/v) Tween-20). The leaves were collected 6 h after the spraying. Leaves sprayed with 0.1% Tween-20 were used as the control.

RNA extraction, cDNA synthesis and real-time quantitative RT-PCR Total RNA from soybean was extracted using Trizol reagent (Invitrogen, USA) and then subjected to RNase-free DNase I (Promega, USA) digestion and purification. First strand cDNA was synthesized using AMV reverse transcriptase (BioFlux, Fluxion Biosciences, Inc., USA). Real-time quantitative PCR (qRT-PCR) was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The amplification reaction was performed in a volume of 25 μ L, which contains approximately 5 ng template, 12.5 μ L SYBR-Green PCR Mastermix (Toyobo Japan) and 10 pmol of each primer. The temperature profile was 98°C for 30 s followed by 40 cycles at 98°C for 2 s, 60°C for 5 s and a melt curve at 65°C for 5 s. Cycle threshold values for each target gene were normalized according to those obtained in the corresponding reactions for the soybean elongation factor gene (accession no. EU790565.1). Relative expression was estimated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The primers used included: 5'-TGGAATGGTGGTGACTTTTGC-3' and 5'-GGTCATCCTTTGAGTTTGAGGC-3' for Gm*EF-1 α* ; 5'-TTCACCACCTTCACATCTT-3' and 5'-CGGTCAGTCTCGTAGTTC-3' for Gm*Mlo4*; 5'-AAGGATAGTTGGTGTGAGT-3' and 5'-CAAGAGATACAGGAATGATAGC-3' for

Gm*Mlo15*; 5'-GAAGA GGAACACCGTAGG-3' and 5'-TGAAGGACTGCTAAGAAGAATA-3' for Gm*Mlo17*; and 5'-ACACCACACCATTCTCAA-3' and 5'-GCACTCTCACTTCTTCCA-3' for Gm*Mlo19*.

RESULTS

Identification of *Mlo* genes in soybean To identify all the putative genes of the *Mlo* gene family in soybean, BLAST was used to search the soybean genome databases (<http://www.phytozome.net/soybean.php>, phytozome) using the HMM Profile of the MLO domain as a query. By using this approach, 20 putative soybean *Mlo* genes were identified. The EST database provided confirmative transcript information for all *Mlo* genes. These genes were designated sequentially from Gm*Mlo1* to Gm*Mlo20*, according to their genome location (Table 1). The information of Gm*Mlos*, including accession numbers of the full-length cDNAs and proteins, and their physical locations on the chromosomes are listed in Table 1. Diverse exon-intron structures were identified by comparing the full-length cDNAs or predicted CDS with the genomic sequence of Gm*Mlo* genes in the soybean genome databases (Fig. 1). Gm*Mlo* exons ranged in number from 13 to 16. There were 11 Gm*Mlo* genes containing a 5' or 3'

Table 1. Summary of Gm*Mlo* family members

Gene name	Locus	Peptide	Gene structure			Domain		Character	
			Number of Exon	Number of intron	UTR	Length	location	TM	CaMBD
Gm <i>Mlo1</i>	01g36170.1	597	14	13	5';3'UTR	478	32-519	7	Yes
Gm <i>Mlo2</i>	02g07110.1	589	16	15	No	507	7-513	6	Yes
Gm <i>Mlo3</i>	03g22960.1	518	13	12	5';3'UTR	497	6-502	7	Yes
Gm <i>Mlo4</i>	04g00370.1	507	13	12	5';3'UTR	489	6-494	7	Yes
Gm <i>Mlo5</i>	04g01710.1	469	16	15	No	448	8-455	5	Yes
Gm <i>Mlo6</i>	04g01730.1	545	14	13	No	490	7-496	7	Yes
Gm <i>Mlo7</i>	06g00440.1	498	15	14	5'UTR	469	8-476	7	Yes
Gm <i>Mlo8</i>	06g01820.1	542	14	13	No	483	7-489	7	Yes
Gm <i>Mlo9</i>	08g20120.1	557	15	14	No	400	3-402	6	Yes
Gm <i>Mlo10</i>	10g35640.1	537	15	14	3'UTR	436	1-436	6	Yes
Gm <i>Mlo11</i>	11g09270.1	601	15	14	3'UTR	422	1-422	8	Yes
Gm <i>Mlo12</i>	11g15920.1	599	15	14	3'UTR	427	1-427	7	Yes
Gm <i>Mlo13</i>	12g07530.1	578	15	14	No	403	1-402	7	Yes
Gm <i>Mlo14</i>	12g29330.1	586	16	15	3'UTR	410	1-411	7	Yes
Gm <i>Mlo15</i>	13g35390.2	544	15	14	3'UTR	477	11-487	7	Yes
Gm <i>Mlo16</i>	15g13070.1	509	15	14	No	396	3-398	8	No
Gm <i>Mlo17</i>	16g21510.1	577	15	14	5';3'UTR	412	1-412	8	Yes
Gm <i>Mlo18</i>	16g26090.1	623	16	15	No	436	16-451	7	Yes
Gm <i>Mlo19</i>	16g26100.1	592	16	15	3'UTR	402	12-413	7	Yes
Gm <i>Mlo20</i>	20g31910.1	560	15	14	No	376	1-376	6	Yes

UTR, Untranslated region; TM, Transmembrane segments; CaMBD, CaM-binding domain.

Fig. 1. Intron-exon organization of 20 *GmMlo* genes. Exon(s) are shown in black and spaces between the black boxes correspond to introns.

Fig. 2. Genomic distribution of *GmMlo* genes on soybean chromosomes. The bars represent the loci with the gene name on the right. A white circle on the chromosome indicates the approximate position of the centromeres. Chromosome numbers are indicated at the bottom of each chromosome.

un-translated region (UTR). The 20 *GmMlo* genes were unevenly located on the 13 chromosomes (Fig. 2) and the number of genes ranged from one to three ranged per chromosomes. There was only one group, *GmMlo18/19* that had a clustered distribution on the soybean chromosome, whereas the remainder had a scattered distribution. Meanwhile, nine *GmMlo* genes including *GmMlo1*, 11, 17, *GmMlo2*, 3, 18 and *GmMlo12*, 13, 14 were identified on segmental duplicated regions.

Sequence analysis of GmMLO proteins GmMLO proteins have a typically MLO domain. The length of the MLO proteins varied greatly, from a minimum of 469 (GmMLO5) to 623 amino acids (GmMLO18) (Table 1). The multiple alignments of the amino acids of GmMLOs showed considerable sequence diversity and the overall pairwise sequence identity was generally

<60% (data not shown). However, more identity was found among the MLO domain of the GmMLO proteins (Fig. 3).

TM structures were predicted by using TMHMM2 and showed that the GmMLO proteins contained five to eight TM segments (Table 1); these putative TM segments were located on GmMLOs, based on sequence alignment (Fig. 3). An exact match for CaMBD, an important motif for the calcium response, was found on the C-terminal cytoplasmic tail of all the GmMLO proteins, with the exception of GmMLO16. Highly conserved tryptophan (Trp) residues and hydrophobic residues located at positions 1, 8 and 14 were observed in the CaMBD-containing GmMLO proteins (Fig. 3).

Phylogenetic analysis of MLO proteins To gain insight into the potential function of different GmMLO pro-

Fig. 3. Sequence alignments of GmMLO proteins. Dark shading indicates invariant residues. Black or white triangles indicate non-variable Trp residues and highly conserved hydrophobic amino acids in the CaMBD domain.

teins, a phylogenetic tree was constructed to compare the relationship between MLOs from other plant species (20 in soybean; 15 in *Arabidopsis*; 9 in maize; 11 in rice (but

not OsMLO10); 3 in barley; 6 in wheat; and 3 MLO proteins from other species) (Fig. 4). The results showed that the MLO family comprises four subfamilies and a

Fig. 4. Phylogenetic analysis of MLO proteins from soybean and other plant species. The *Arabidopsis* CRT1 protein (NP_001031199) was selected as the outgroup. The tree shows the four major phylogenetic subfamilies (numbered I to IV and marked with different alternating background to make subfamily identification easier) with high predictive value. At, *Arabidopsis thaliana*; Br, *Brassica rapa*; Ca, *Capsicum annuum*; Hv, *Hordeum vulgare*; Le, *Lycopersicon esculentum*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Ta, *Triticum aestivum*; Zm, *Zea mays*.

single divergent lineage (AtMLO3). Subfamilies III and IV showed strong bootstrap support for a sister group relationship, and contained in total, 14 GmMlo genes. Four GmMlo genes (GmMlo1, 5, 11, 17) belonged to subfamily III and ten GmMlo genes (GmMlo2, 6, 8, 10, 12, 13, 14, 18, 19, 20) belonged to subfamily IV. Other GmMlo9 and GmMlo15 belonged to subfamily I and four GmMlo genes (GmMlo3, 4, 7, 16) belonged to subfamily II.

Expression of GmMlos under abiotic stresses and phytohormone applications To find out whether GmMlos are response to abiotic stresses, the expression of GmMlo genes was examined in plants subjected to abiotic treatments (heat, cold, drought and salinity). One GmMlo gene was selected from each subfamily. The expression of four GmMlo genes (GmMlo4, 15, 17, 19) was analyzed (Fig. 5). After heat treatment, the transcription levels of four genes were downregulated (Fig. 5A). Under cold conditions, the expression of GmMlo15 and GmMlo17 was upregulated, whereas that of GmMlo19 was downregulated; however, this treatment had no effect on GmMlo4 (Fig. 5B). As shown in Fig. 5C, GmMlo4 was upregulated under drought conditions, whereas the other three genes were downregulated (Fig. 5C). GmMlo15 and GmMlo17 were downregulated under the salinity treatment, although, this treatment had no effect on either GmMlo4 or GmMlo19 (Fig. 5D).

Given that the abiotic stress response involves phytohormone signaling pathways, the expression of GmMlo genes was examined in response to exogenous application of ABA, 6-BA, IAA and GA₃. As shown in Fig. 5 (E and F), four genes were suppressed in leaves were sprayed with either 10 mM ABA or 1 mM 6-BA. GmMlo4 and GmMlo15, 17, and 19 expressions was also up- or down-regulated in leaves sprayed with 1 mM IAA (Fig. 5G). GmMlo15 showed no response in leaves sprayed with 10 mM GA₃, whereas GmMlo4 and GmMlo17 expression was induced, but that of GmMlo19 was downregulated (Fig. 5H). The strong response of GmMlos following abiotic stress and phytohormone treatments suggests that they have various roles in the responses of environmental stimuli.

cis-response element analysis of the promoters of GmMlo genes The promoter region of the GmMlo genes (2,0-kb upstream of the transcriptional start site) was analyzed using the Plant CARE database to identify putative stress-responsive cis-elements. Sequence analysis identified a total of nine types of stress-related cis-regulatory elements among GmMlo genes (Table 2). The defense-responsive cis-regulatory elements contained a putative TC-rich repeats element. Abiotic responsive cis-elements, such as the heat-stress element (HSE), were identified in 12 and 16 promoters of GmMlo genes. The drought responsiveness element, which contains MYB

Fig. 5. Expression of GmMlo genes in response to abiotic treatments and phytohormone applications. (A) heat treatment, (B) cold treatment, (C) drought treatment, (D) salinity treatment, (E) ABA treatment, (F) 6-BA treatment, (G) IAA treatment and (H) GA3 treatment. '0' represents a simulated treatment. The horizontal axis represents the time (h) after either abiotic or phytohormone treatments. Error bars represent the standard deviation from three replicated experiments.

Table 2. Putative regulatory *cis*-element sequences in the promoter of GmMlo genes

Element name	Defense		Heat	Drought		Auxin	ABA	Gibberellin
	TC-rich repeats	HSE	MYB binding site/ DRE	AuxRE	ABRE	GARE-motif/ TATC-box/ P-box		
GmMlo1	+	+	+			+		
GmMlo2	+	+	+					+
GmMlo3		+	+	+				
GmMlo4	+				+	+		
GmMlo5	+		+		+			
GmMlo6	+	+	+			+		
GmMlo7	+	+						
GmMlo8		+	+		+			
GmMlo9			+		+	+		
GmMlo10	+	+			+			
GmMlo11		+	+		+			
GmMlo12		+						
GmMlo13		+			+			
GmMlo14	+	+	+	+	+			
GmMlo15	+		+			+		
GmMlo16	+	+	+			+		
GmMlo17	+	+	+		+	+		
GmMlo18	+	+	+		+			
GmMlo19		+	+		+			
GmMlo20		+						

binding site and a dehydration responsive element (DRE), was identified in 14 promoters of GmMlo genes; The ABA responsive element (ABRE), gibberellin-responsive element (containing a GARE-motif, TATC-box and P-box) and auxin-responsive element (AuxRE) were also identified in 12, 7 and 2 promoters of GmMlo genes, respectively (Table 2).

Major stress-responsive genes contain putative corresponding stress-responsive *cis*-elements in their promoters. For example, the GA₃-responsive element was identified in the promoter region of GmMlo4 and GmMlo17, which were induced by GA₃ treatment, GmMlo19 were also induced in response to GA₃ treatments, and the corresponding elements was identified in its promoters. However, the putative *cis*-elements detected in GmMlo genes were not closely related with the stress responsiveness. GARE was identified in the promoter of GmMlo15, but the gene did not show a change in response to GA₃ treatment. ABA-responsive elements were identified in the promoter of GmMlo19, despite the fact that the gene was downregulated after in leaves treated with ABA.

DISCUSSION

Mlo is a multi-membered gene family. So far, 15, 12, 9 and 7 Mlo genes have been identified in *Arabidopsis thaliana*, rice, maize and wheat, respectively. Mean-

while, a few *Mlo* genes have been identified and isolated from barley, tomato *Lycopersicon*, pepper *Capsicum* and cabbage *Brassica*, respectively (Devoto et al., 2003; Miklis et al., 2007; Liu and Zhu, 2008; Konishi et al., 2010). In the current study, 20 MLO domain-containing sequences were identified in soybean, based on database searches and analysis (Table 1). The GmMlos were similar in character to other *Mlo* gene members in *Arabidopsis*, barley and wheat; for example, in terms of their TM and CaMBD structures. The seven TM structures are thought to be a key characteristic of the *Mlo* gene family (Devoto et al., 2003; Elliott et al., 2005; Feechan et al., 2008). Seven TM segments were also located on GmMLO members based on sequence alignment. However, as predicted by TMHMM2, the GmMLO proteins could contain five to eight TM segments (Table 1). The various TM structures might be the result of structural and allosteric complexity that is too difficult to be predicted by computational methods (Devoto et al., 1999; Kenakin, 2009). Therefore, the occurrence of seven TM segments is also thought to be a main characteristic among GmMLO proteins. The CaMBD motif located on the C-terminus in MLO family members has been identified as an important Ca^{2+} signal response motif that modulates defence reactions against powdery mildew (Kim et al., 2002a, 2002b). Among the 20 predicted GmMlo genes, 19 genes have the conserved CaMBD motif. GmMlo6 did not show any detectable CaMBD motif, but it may be a special *Mlo* gene in soybean genome, also, it may be a pseudogene.

GmMlo genes were distributed on 13 soybean chromosomes, with a mainly scattered distribution except one group existed a clustered distribution. Gene families could arise through tandem amplification or segmental duplication of chromosomal regions. Tandem amplification results in a clustered distribution, whereas segmental duplication results in a scattered distribution (Schauser et al., 2005). Therefore, it is suggested that segmental duplication is the main amplification method that occurs in GmMlo family members; in the current study, three groups of GmMlo genes were identified from one segmental duplication event. The result is similar to AtMlos and OsMlos family members, which also mainly result from segmental duplication (Devoto et al., 2003; Liu and Zhu, 2008). Meanwhile, the existence of multiple *Mlo* gene copies in soybean is not surprising, soybean is a paleopolyploid, also, paleopolyploid must have been the factor of gene duplication. Moreover, the relationship of segmental duplication with clustering in the phylogenetic tree was appeared, It seems that members derived from segmental chromosome duplication such as GmMlo1, 11, 17 are clustered together in the same subfamily.

The response of *Mlo* family members to Ca^{2+} signals to modulated plant defence reactions against powdery mil-

dew and to regulated cell death has been identified (Kim et al., 2002a, 2002b; Bai et al., 2008). Increasing evidences suggests that *Mlo* genes have additional functions. Their transcription can occur in response to wounding, paraquat, and auxins and phytohormone stress (Piffanelli et al., 2002; Feechan et al., 2008). In the current study, four GmMlos were responsive to various abiotic stresses and phytohormone treatments. Multi-type abiotic- and phytohormone-responsive elements predicted in the promoters of the GmMlo genes were also take as evidence of the various stress-responsive functions of GmMlos. The ABA-, gibberellin- and auxin-responsive elements, in particular, were identified in parts of promoters of GmMlo genes. ABA is an important phytohormone that can convert the initial stress signal, such as drought or high salinity into a cellular response (Fujita et al., 2005; Nakashima et al., 2009). Auxins and gibberellins are also important signal molecules in environmental signal transduction (Sun, 2000; Berleth et al., 2004). In addition, crosstalk between phytohormones and Ca^{2+} signals in response to various environmental stresses has been reported (Van der Meulen et al., 1996; Gao et al., 2002; Yang and Poovaiah, 2003; Navarro-Avino and Bennet, 2005; Qudeimat et al., 2008; Siegel et al., 2009; Kang et al., 2010; Zou et al., 2010). However, this evidence comes mainly from *Arabidopsis*. To date, many questions remain relating to the mechanisms of MLO proteins. Crosstalk between abiotic stresses and phytohormone signaling pathways increases the complexity of MLO function. Isolation and characterization of *Mlo* genes on a genome scale could pave the way for functional investigations. The results from the current study provide obvious clues for further elucidating the functions of members of the *Mlo* genes family in response to environmental stimuli.

This work was partially supported by Research Fund for the Doctoral Program of Higher Education of China (No.20090097120023) and Science and Technology Foundation of Guizhou province (No. [2010] 2089).

REFERENCES

- Bai, Y., Pavan, S., Zheng, Z., Zappel, N. F., Reinstädler, A., Lotti, C., De Giovanni, C., Ricciardi, L., Lindhout, P., Visser, R., et al. (2008) Naturally occurring broad-spectrum powdery mildew resistance in a Central American tomato accession is caused by loss of *Mlo* function. *Mol. Plant-Microbe Interact.* **21**, 30–39.
- Berleth, T., Krogan, N. T., and Scarpella, E. (2004) Auxin signals-turning genes on and turning cells around. *Curr. Opin. Plant Biol.* **7**, 553–563.
- Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., et al. (1997) The barley *Mlo* gene: A novel control element of plant pathogen resistance. *Cell* **88**, 695–705.
- Consonni, C., Humphry, M. E., Hartmann, H. A., Livaja, M.,

- Durner, J., Westphal, L., Vogel, J., Lipka, V., Kemmerling, B., Schulze-Lefert, P., et al. (2006) Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. *Nat. Genet.* **38**, 716–720.
- Consonni, C., Bednarek, P., Humphry, M., Francocci, F., Ferrari, S., Harzen, A., Ver Loren van Themaat, E., and Panstruga, R. (2010) Tryptophan-derived metabolites are required for antifungal defense in the *Arabidopsis mlo2* mutant. *Plant Physiol.* **152**, 1544–1561.
- Devoto, A., Piffanelli, P., Nilsson, I., Wallin, E., Panstruga, R., von Heijne, G., and Schulze-Lefert, P. (1999) Topology, subcellular localization, and sequence diversity of the *Mlo* family in plants. *J. Biol. Chem.* **274**, 34993–35004.
- Devoto, A., Hartmann, H. A., Piffanelli, P., Elliott, C., Simmons, C., Taramino, G., Goh, C. S., Cohen, F. E., Emerson, B. C., Schulze-Lefert, P., et al. (2003) Molecular phylogeny and evolution of the plant-specific seven-transmembrane MLO family. *J. Mol. Evol.* **56**, 77–88.
- Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S., and Pooviah, B. W. (2009) Ca^{2+} /calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* **457**, 1154–1158.
- Elliott, C., Müller, J., Miklis, M., Bhat, R. A., Schulze-Lefert, P., and Panstruga, R. (2005) Conserved extracellular cysteine residues and cytoplasmic loop-loop interplay are required for functionality of the heptahelical MLO protein. *J. Biochem.* **385**, 243–254.
- Feechan, A., Jermakow, A. M., Torregrosa, L., Panstruga, R., and Dry, I. B. (2008) Identification of grapevine *MLO* gene candidates involved in susceptibility to powdery mildew. *Funct. Plant Biol.* **35**, 1255–1266.
- Finn, R. D., Mistry, J., Schuster-Böckler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khanna, A., Durbin, R., et al. (2006) Pfam: clans, web tools and services. *Nucleic Acids Res.* **34** (Suppl. 1), D247–D251.
- Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M. M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2005) AREB1 is a transcription activator of novel ABRE-dependent ABA-signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* **17**, 3470–3488.
- Gao, Y. P., Bonham-Smith, P. C., and Gusta, L. V. (2002) The role of peroxiredoxin in antioxidant and calmodulin ABA-primed seeds of *Brassica napus* exposed to abiotic stresses during germination. *J. Plant Physiol.* **159**, 951–958.
- Hrabak, E. M., Chan, C. W. M., Gribskov, M., Harper, J. F., Choi, J. H., Halford, N., Kudla, J., Luan, S., Nimmo, H. G., Sussman, M. R., et al. (2003) The *Arabidopsis* CDPK-SnRK superfamily of protein kinases. *Plant Physiol.* **132**, 666–680.
- Jørgensen, I. H. (1992) Discovery, characterization and exploitation of *Mlo* powdery mildew resistance in barley. *Euphytica* **63**, 141–152.
- Kang, S. M., Kim, J. T., Hamayun, M., Hwang, I. C., Khan, A. L., Kim, Y. H., Lee, J. H., and Lee, I. J. (2010) Influence of prohexadione-calcium on growth and gibberellins content of Chinese cabbage grown in alpine region of South Korea. *Scientia Horticulturae* **125**, 88–92.
- Kenakin, T. P. (2009) 7 TM Receptor Allosterity: Putting numbers to shape shifting proteins. *Trends Pharmacol. Sci.* **30**, 460–469.
- Kim, M. C., Lee, S. H., Kim, J. K., Chun, H. J., Choi, M. S., Chung, W. S., Moon, B. C., Kang, C. H., Park, C. Y., Yoo, J. H., et al. (2002a) Mlo, a modulator of plant defense and cell death, is a novel calmodulin-binding protein. *J. Biol. Chem.* **277**, 19304–19314.
- Kim, M. C., Panstruga, R., Elliott, C., Müller, J., Devoto, A., Yoon, H. W., Park, H. C., Cho, M. J., and Schulze-Lefert, P. (2002b) Calmodulin interacts with MLO protein to regulate defence against mildew in barley. *Nature* **416**, 447–450.
- Konishi, S., Sasakuma, T., and Sasanuma, T. (2010) Identification of novel *Mlo* family members in wheat and their genetic characterization. *Gene Genet. Syst.* **85**, 167–175.
- Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) Predicting transmembrane protein topology with hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**, 567–580.
- Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P., and Rombauts, S. (2002) PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acid Res.* **30**, 325–327.
- Letunic, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P., and Bork, P. (2004) SMART4.0: towards genomic data integration. *Nucleic Acids Res.* **32**, 142–144.
- Liu, Q., and Zhu, H. (2008) Molecular evolution of the *MLO* gene family in *Oryza sativa* and their functional divergence. *Gene* **409**, 1–10.
- Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\text{CT}}$ method. *Methods* **25**, 402–408.
- Miklis, M., Consonni, C., Bhat, R. A., Lipka, V., Schulze-Lefert, P., and Panstruga, R. (2007) Barley MLO modulates actin-dependent and actin-independent antifungal defense pathways at the cell periphery. *Plant Physiol.* **144**, 1132–1143.
- Nakashima, K., Ito, Y., and Yamaguchi-Shinozaki, K. (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant Physiol.* **149**, 88–95.
- Navarro-Aviñó, J. P., and Bennett, A. B. (2005) Role of a Ca^{2+} -ATPase induced by ABA and IAA in the generation of specific Ca^{2+} signals. *Biochem. Biophys. Res. Commun.* **329**, 406–415.
- Piffanelli, P., Zhou, F., Casais, C., Orme, J., Jarosch, B., Schaffrath, U., Collins, N. C., Panstruga, R., and Schulze-Lefert, P. (2002) The barley *MLO* modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. *Plant Physiol.* **129**, 1076–1085.
- Piffanelli, P., Ramsay, L., Waugh, R., Benabdelmouna, A., D'Hont, A., Hollricher, K., Jørgensen, J. H., Schulze-Lefert, P., and Panstruga, R. (2004) A barley cultivation-associated polymorphism conveys resistance to powdery mildew. *Nature* **430**, 887–891.
- Qudeimat, E., Faltusz, A. M. C., Wheeler, G., Lang, D., Holtorf, H., Brownlee, C., Reski, R., and Frank, W. (2008) A PIIB-type Ca^{2+} -ATPase is essential for stress adaptation in *Physcomitrella patens*. *Proc. Acad. Natl. Sci. USA.* **105**, 19555–19560.
- Reddy, A. S. (2001) Calcium: silver bullet in signaling. *Plant Sci.* **160**, 381–404.
- Rudd, J. J., and Franklin-Tong, V. E. (2001) Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells. *New Phytol.* **151**, 7–33.
- Sanders, D., Pelloux, J., Brownlee, C., and Harper, J. F. (2002) Calcium at the crossroads of signaling. *Plant Cell* **14**, 401–417.
- Schauser, L., Wieloch, W., and Stougaard, J. (2005) Evolution of NIN-Like proteins in *Arabidopsis*, rice, and *Lotus japonicus*. *J. Mol. Evol.* **60**, 229–237.

- Siegel, R. S., Xue, S., Murata, Y., Yang, Y., Nishimura, N., Wang, A., and Schroeder, J. I. (2009) Calcium elevation-dependent and attenuated resting calcium-dependent abscisic acid induction of stomatal closure and abscisic acid-induced enhancement of calcium sensitivities of S-type anion and inward-rectifying K channels in *Arabidopsis* guard cells. *Plant J.* **59**, 207–220.
- Stein, M., and Somerville, S. C. (2002) MLO, a novel modulator of plant defenses and cell death, binds calmodulin. *Trends Plant Sci.* **7**, 379–380.
- Sun, T. P. (2000) Gibberellin signal transduction. *Curr. Opin. Plant Biol.* **3**, 374–380.
- Van der Meulen, R. M., Visser, K., and Wang, M. (1996) Effects of modulation of calcium levels and calcium fluxes on ABA-induced gene expression in barley aleurone. *Plant Sci.* **117**, 75–82.
- Wang, L., Tsuda, K., Sato, M., Cohen, J. D., Katagiri, F., and Glazebrook, J. (2009) *Arabidopsis* CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. *PLoS Pathog.* **5**, e1000301.
- Wolter, M., Hollricher, K., Salamini, F., and Schulze-Lefert, P. (1993) The *mlo* resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defense mimic phenotype. *Mol. Gen. Genet.* **239**, 122–128.
- Yang, T., and Poovaiah, B. W. (2003) Calcium/calmodulin-mediated signal network in plants. *Trends Plant Sci.* **8**, 505–512.
- Zielinski, R. E. (1998) Calmodulin and calmodulin-binding proteins in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 697–725.
- Zou, J. J., Wei, F. J., Wang, C., Wu, J. J., Ratnasekera, D., Liu, W. X., and Wu, W. H. (2010) *Arabidopsis* calcium-dependent protein kinase CPK10 functions in abscisic acid- and Ca^{2+} -mediated stomatal regulation in response to drought stress. *Plant Physiol.* **154**, 1232–1243.