Identification and Characterization of a Potential Candidate *Mlo* Gene Conferring Susceptibility to Powdery Mildew in Rubber Tree

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ABSTRACT

Mildew resistance locus O (*Mlo*) gene was first found in barley as a powdery mildew susceptibility gene, and recessive *mlo* alleles confer durable resistance to barley powdery mildew. To identify candidate *Mlo* susceptibility genes in rubber tree, *HbMlo12* was cloned from rubber tree clone CATAS7-33-97, which is susceptible to powdery mildew. Protein architecture analysis showed that *HbMlo12* was a typical *Mlo* protein with seven transmembrane domains. Protein blast search in the *Arabidopsis thaliana* proteome database showed that *HbMlo12* shared the highest similarity with *AtMlo12*, with 63% sequence identity. Furthermore, *HbMlo12* together with the dicot powdery mildew susceptible *Mlo* proteins (including *AtMlo2*, *AtMlo6*, *AtMlo12*, tomato *SlMlo1*, pepper *CaMlo2*, pea *PsMlo1*, etc.) were grouped into clade V. Subcellular localization analysis in tobacco epidermal cells revealed that *HbMlo12* was localized to the endoplasmic reticulum membrane. *HbMlo12* was preferentially expressed in the flower and leaf of rubber

tree. Moreover, its expression was significantly upregulated in response to powdery mildew inoculation. Application of exogenous ethephon caused a distinct increase in *HbMlo12* expression. Additionally, *HbMlo12* transcript was quickly induced by spraying salicylic acid and gibberellic acid and reached the maximum at 0.5 h after treatments. By contrast, *HbMlo12* expression was downregulated by methyl jasmonate, abscisic acid, and drought stress treatments. There was no significant change in *HbMlo12* expression after indole-3-acetic acid, H₂O₂, and wounding stimuli. Taken together, these results suggested that *HbMlo12* might be a candidate *Mlo* gene conferring susceptibility to powdery mildew in rubber tree. The results of this study are vital in understanding *Mlo* gene evolution and developing new rubber tree varieties with powdery mildew resistance using reverse genetics.

Keywords: *Hevea brasiliensis*, *Mlo*, powdery mildew, phytohormone

Mildew resistance locus O (*Mlo*) protein is a plant-specific protein family. This gene family is reported in few mosses, but it is ubiquitous in all higher plants (Büschges et al. 1997). *Mlo* was first found in barley (*Hordeum vulgare*) as a powdery mildew susceptibility gene. *HvMlo* had been isolated by map-based cloning and encoded a protein with “hairpin structure” of seven transmembrane domains, which was regarded as its “topological signature” (Büschges et al. 1997; Devoto et al. 1999). *Mlo* proteins are characterized by conserved seven transmembrane regions and C-terminal calmodulin (CaM) binding domains (Kim et al. 2002b). Subcellular localization studies showed that *Mlo* proteins were located in the plasma membrane with an extracellular amino terminus and an intracellular carboxy terminus (Devoto et al. 1999, 2003; Kim and Hwang 2012). The second and third cytoplasmic loops are critical for the susceptibility-conferring activity of *HvMlo* (Reinstadler et al. 2010). Wild-type *HvMlo* suppresses oxidative burst and cell death at mesophyll cells, which are often associated with plant pathogen resistance. Moreover, *HvMlo* expression was upregulated in response to powdery mildew inoculation and under a

range of biotic and abiotic stress conditions. These data imply that *HvMlo* plays a broad role in cell death protection, defense, and stress response processes (Piffanelli et al. 2002). Loss of function of the *Mlo* gene (*mlo*) confers durable and broad-spectrum resistance to almost all known isolates of powdery mildew in barley (Büschges et al. 1997). The *mlo*-mediated resistance is durable under the field conditions, and it has been used to develop powdery mildew-resistant barley varieties that have been cultivated in the disease-prone areas (Jørgensen 1992).

In addition to barley, *Mlo* has been identified in other plant species. The number of *Mlo* is 15 in the genus *Arabidopsis* (Consonni et al. 2006), 14 in cucumber (Zhou et al. 2013), 17 in grape (Feechan et al. 2009), 16 in tomato (Bai et al. 2008), 13 in rice (Liu and Zhu 2008), 7 in wheat (Konishi et al. 2010), 21 in apple, 19 in peach, and 18 in strawberry (Pessina et al. 2014). These plant *Mlo* gene families have been classified into at least seven clades (Acevedo-Garcia et al. 2014; Kusch et al. 2016; Pessina et al. 2014). All *Mlo* proteins from dicot species that are known to be associated with powdery mildew susceptibility sort into clade V (including *AtMlo2*, *AtMlo6*, *AtMlo12*, tomato *SlMlo1*, pea [*Pisum sativum*] *Er1/PsMlo1*, *CsaMLO8*, etc.), and monocot *Mlo* proteins sort into clade IV (including *HvMlo*, wheat *TaMlo1A*, *TaMlo1B*, and *TaMlo1D*). *Mlo* proteins involved in powdery mildew interaction seem to be restricted to these two clades (Acevedo-Garcia et al. 2014; Berg et al. 2015; Consonni et al. 2006; Elliott et al. 2002; Kim et al. 2002b; Wang et al. 2014;). Some of them have confirmed that powdery mildew resistance is derived from loss-of-function mutations in functional *Mlo* orthologs. For example, mutant alleles of *AtMlo2* in *Arabidopsis thaliana* caused partial resistance to the adapted strains, such as *Golovinomyces orontii* and *Golovinomyces cichoracearum*. The *Atmlo2 Atmlo6 Atmlo12* triple mutant confers complete powdery mildew resistance, and powdery mildew fails to infect the genus *Arabidopsis* at the early stage in the triple-mutant

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plants. *AtMlo2*, *AtMlo6*, and *AtMlo12* share partially redundant functions in susceptibility to *Arabidopsis* powdery mildews (Consonni et al. 2006). Unlike the genus *Arabidopsis*, loss of function of a single *Mlo* gene in tomato (*SlMlo1*) suffices to confer full powdery mildew resistance (Bai et al. 2008). The loss of *PsMlo1* (*Er1*) function also gives durable broad-spectrum powdery mildew resistance in pea (Humphry et al. 2011). Silencing of either pepper (*Capsicum annuum*) *CaMlo1* or *CaMlo2* can reduce the susceptibility of pepper to *Leveillula taurica* (Zheng et al. 2013). Other than functioning in modulating antifungal defense, studies revealed that *Mlo* participates in a variety of cellular functions. *Mlo* genes are involved in diverse abiotic stresses in pepper (Kim and Hwang 2012). *CaMlo2* was intensely induced on exogenous treatment of pepper leaves with abscisic acid (ABA) and drought stress, which acted as a suppressor of ABA signaling to prevent water loss from leaves under drought conditions (Lim and Lee 2014). Conservation of *mlo* resistance across monocot and dicot plant species implicates a common mechanistic basis for this type of plant immunity (Acevedo-Garcia et al. 2014). Therefore, inactivation of the *Mlo* susceptibility gene is a valid strategy to introduce powdery mildew resistance in many agriculturally and economically important plant species. In bread wheat, mutation of all three *Mlo* alleles created by TALEN and CRISPR-Cas9 confers heritable broad-spectrum resistance to powdery mildew (Wang et al. 2014).

Rubber tree (*Hevea brasiliensis* [Willd. ex A. Juss.] Müll. Arg.) is the most important source of natural rubber (Bandurski and Teas 1957). Rubber tree growth and natural rubber production are affected by many factors, such as pathogen infection, drought stress, frequent tapping for latex harvest, and ethephon (ET; releasing ethylene) treatments in the bark of stem for stimulating latex regeneration and flow. Powdery mildew is an important disease of rubber trees caused by *Oidium heveae* B. A. Steinm. Rubber tree powdery mildew affects young rubber leaves, buds, inflorescences, and other immature tissues, reducing rubber latex yields by up to 45% (Liyanage et al. 2016). Although some newly bred rubber clones have shown resistance to this disease, no powdery mildew-resistant gene has been isolated in rubber tree. Because mutations of *Mlo* proteins from the clade V conferring powdery mildew resistance in dicot plants have been reported, none of *Hevea Mlo* genes isolated were grouped into this clade. To identify susceptible *Mlo* genes in rubber tree, protein sequences of *Arabidopsis AtMlo2*, *AtMlo6*, and *AtMlo12* were used for tblastn searching in the transcriptome shotgun assembly (TSA) database of rubber tree. A TSA sequence (GenBank accession number JT925277; named as *HbMlo12* in this study) showed the highest similarity with *AtMlo12*. Moreover, phylogenetic analysis of five other *Mlos* from rubber tree (*HbMlo1*, *HbMlo1-1*, *HbMlo7*, *HbMlo8*, and *HbMlo9*) (Qin et al. 2015) and *Arabidopsis Mlos* revealed that *HbMlo12* together with *AtMlo2*, *AtMlo6*, and *AtMlo12* were sorted into clade V. Therefore, in this study, *HbMlo12* was cloned and characterized systematically, and its transcriptional response to powdery mildew infection suggested a candidate for mediating powdery mildew susceptibility in rubber tree.

MATERIALS AND METHODS

Plant materials and treatments. Rubber tree susceptible clone CATAS7-33-97 (Li et al. 2016) was planted at the experimental farm at the Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou City, Hainan Province, China (19°51'51 N; 109°55'63 E). Samples of different tissues (including root, stem, leaf, bark, latex, and flower) were collected from 10-year-old healthy tapping trees. Tobacco (*Nicotiana benthamiana*) was used for transient expression.

Grafted budding seedlings of CATAS7-33-97 were used for multiple stress treatments. For powdery mildew inoculation, seedlings of CATAS7-33-97 with no powdery mildew infection were

grown in an isolated growth chamber at 25°C in a 16-h light/8-h dark cycle at a relative humidity of 85 ± 5%. Tender leaves in the pale green period were used for inoculation and harvested for gene expression analysis. Inoculation of rubber tree powdery mildew was conducted by dusting the conidia from infected young leaves carrying fresh powdery mildew spore. Drought stress, wounding, and chemical treatments were performed according to our previous reports (Qin et al. 2015; Wang 2014). Briefly, seedlings with fully expanded leaves in the dark green period were treated with 5 mM SA, 3 mM GA, 200 µM MeJA, 1.0% (vol/vol) ET (an ethylene releasing compound), 200 µM ABA, 100 µM IAA, or 2% (vol/vol) H₂O₂. For drought stress treatment, seedlings were subjected to progressive drought by withholding water. All of the chemicals were diluted in distilled water containing 0.05% (vol/vol) ethanol. Seedlings treated with 0.05% (vol/vol) ethanol were used as the control for chemical treatments, the well-watered plants were used as the control for drought stress treatment, and the unwounded plants were used as the control for wounding treatment. All of the harvested samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Isolation of genomic DNA and total RNA and first-strand cDNA synthesis. The genomic DNA was extracted from the leaves of rubber tree with the E.Z.N.A. HP Plant DNA Kit (Omega). Total RNA was extracted with the RNAPrep Pure Plant Kit (polysaccharides and polyphenolics rich; TIANGEN Corporation). The quality and concentration of the extracted RNA were checked by agarose gel electrophoresis and measured using a spectrophotometer (Thermo Scientific NanoDrop 2000 Spectrophotometer). First-strand cDNA was synthesized from 2 µg of total RNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions.

Cloning HbMlo12 in rubber tree. The protein sequence of *AtMlo12* (AT2G39200) was used for tblastn searching in the TSA database of rubber tree. A TSA sequence (GenBank accession number JT925277) derived from rubber tree clone RRIM 600 contained the ORF of *Mlo12*. Primers HbMlo12-F and HbMlo12-R (Table 1) were designed flanking the ORF, and they were used to amplify *HbMlo12* by RT-PCR with the leaf cDNA of CATAS7-33-97 as the template. The same primers were also used to clone the genomic sequence of *HbMlo12*. All of the polymerase chain reaction (PCR) products were cloned into pMD18-T vector and sequenced.

Protein architecture and phylogenetic analyses. The theoretical isoelectric point and molecular weight of HbMlo12 were calculated using the ProtParam tool on the ExPASy server (https://web.expasy.org/compute_pi/). NCBI conserved domain search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and SMART (<http://smart.embl-heidelberg.de/>) (Letunic et al. 2004) were used to identify architecture of HbMlo12. The topology of HbMlo12 was analyzed via TOPCONS 2.0 (<http://topcons.cbr.su.se/pred/>) (Tsirigos et al. 2015). Prediction of subcellular localization of HbMlo12 was performed using the PSORT Prediction (<https://psort.hgc.jp/form.html>). Two or multiple sequence alignments were performed using ClustalX 1.83 and DNAMAN 8.0 (Thompson et al. 2003). Phylogenetic analysis was conducted and viewed using MEGA version 6.06 based on the neighbor-joining method with a Poisson correction model and a bootstrap test of 1,000 replicates (Tamura et al. 2013).

Expression analysis of HbMlo12 by real-time quantitative PCR. Specific primers of *HbMlo12* (*HbMlo12*-QF and *HbMlo12*-QR) (Table 1) were used for quantitative RT-PCR amplification, and *HbActin* (GenBank accession number HO004792; *HbActin*-F and *HbActin*-R) (Table 1) was used as the housekeeping gene. The PCR reaction was performed in a 20-µl reaction mixture containing 1× SYB Premix Ex Taq (Takara), 0.2 µmol liter⁻¹ each primer, and about 30 ng cDNA per sample using the CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad). The reaction program was as follows: 3 min at 95°C for denaturation

followed by 45 cycles of 10 s at 95°C, 20 s at 60°C, and 30 s at 72°C; subsequently, a melting curve was generated for the PCR product. The samples were cooled to 60°C (20°C per second) followed by heating in steps of 0.2°C per second up to 95°C. After completion of the reaction, the cycle threshold (C_T) value was calculated, and then, the $\Delta\Delta C_T$ algorithm was used to calculate the relative expression level of target gene (Livak and Schmittgen 2001).

Subcellular localization of HbMlo12 using *Agrobacterium*-mediated transient expression in tobacco. Identification of subcellular localization of HbMlo12 was using the 35S:XXGFP fusion protein of mGFP with confocal laser microscopy. The 35S:XXGFP vector was provided by Xing-liang Hou (South China Botanical Garden, Chinese Academy of Sciences, Guangzhou,

China); 35S:XXGFP with an enhanced CaMV 35S promoter at the N terminus of multiple cloning sites (MCSs) and an mGFP at the C terminus of MCSs was used for transient expression analysis in tobacco. The 35S:XXGFP vector was produced from pGreen 0229 IT by insertion of mGFP-6xHis between *SpeI* and *XbaI* restriction sites. The mGFP is amplified from pMDC83 plasmid and contains a

Fig. 2. Splicing feature of *HbMlo12* in susceptible rubber tree clone CATAS7-33-97. The exons are indicated by boxes, and introns are indicated by thick lines.

Fig. 1. Multiple amino acid sequence alignment of HbMlo12, AtMlo2, AtMlo6, and HvMlo. The seven transmembrane domains (TM1 to TM7) of HbMlo12 are indicated by lines above the sequences. The C-terminal D/E-F-S/T-F tetrapeptide sequences are indicated by asterisks. Three distinctive regions (motifs 25, 26, and 27) identified in the mildew resistance locus O (Mlo) associated with powdery mildew susceptibility are shown in the boxes.

6xHis tag at the C terminus. The ORF of *HbMlo12* was amplified by primers *HbMlo12-XhoI-F* and *HbMlo12-BamHI-R* (Table 1) and subcloned via *XhoI/BamHI* into the 35S:XXGFP vector. The generated 35S:HbMlo12:mGFP plasmid was verified by sequencing and transformed into *Agrobacterium tumefaciens* strain GV3101. For transient expression, the *Agrobacterium* strain GV3101 with the target gene expression cassette 35S:HbMlo12:mGFP was cotransformed into tobacco leaf epidermal cells by infiltration (asexis liquor with final concentration of 28 mmol·liter⁻¹ of glucose, 50 mmol·liter⁻¹ of MES, 3.8 mmol·liter⁻¹ of Na₃PO₄·2H₂O, and 200 μmol·liter⁻¹ of

acetosyringone). The construct expresses HbMlo12-fused mGFP under the control of the CaMV 35S promoter. After infiltration, plants were grown for 24 h in darkness and transferred to light for 48 h before detection of the GFP signals. The GFP fluorescence was determined using Leica TCS SP8 MP laser confocal fluorescence microscopy (Leica Microsystems Inc.).

Statistical analysis. For each sample, quantitative RT-PCR was performed with three technical replicates and three biological replicates. The 2^{-ΔΔCT} method was used to analyze relative expression levels of the target gene (Nolan et al. 2006; Schmittgen and

Fig. 3. Phylogenetic analysis of *HbMlo12* with other mildew resistance loci O (Mlos). The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. The phylogenetic tree was constructed via MEGA 6.06. The color code associated with the proteins was grouped into the same clade. *HvMlo1* (syn. *HvMlo*), *TaMlo1A*, *TaMlo1B*, *TaMlo1D*, *AtMlo2*, *AtMlo6*, *AtMlo12*, *SIMLO6* (syn. *SIMlo1*), *CsMLO5* (syn. *CsaMLO8*) are involved in susceptibility to powdery mildew (Bai et al. 2008; Berg et al. 2015; Büschges et al. 1997; Consonni et al. 2006; Wang et al. 2014).

Fig. 4. Topology prediction of HbMlo12. **A**, The subcellular localization of HbMlo12 was predicted using online analysis tool PSORT prediction. **B**, The transmembrane region of HbMlo12 was predicted using the TOPCONS 2.0 prediction server.

Fig. 5. Subcellular localization of HbMlo12. Merge indicates the merge of the green fluorescence protein (GFP) image and the bright-field (DIC) image.

Livak 2008). The data from quantitative RT-PCR were analyzed with IBM-SPSS analytical software package version 24.0 (IBM Corporation). The data for replicates were analyzed by one-way analysis of variance, and multiple comparisons were performed by Duncan test at $P < 0.05$. The *HbMlo12* expression in the tested samples was firstly calculated relative to that in control plants (seedlings treated with 0.05% [vol/vol] ethanol were used as the control for chemical treatment, the well-watered plants were used as the control for drought stress treatment, and the unwounded plants were used as the control for wounding treatment). The final value of relative gene quantification was indicated as n -fold change in comparison with the untreated control (0 h). Figures were drawn by Origin data analysis and graphing software OriginPro 2016 (OriginLab Corporation).

RESULTS

Cloning *HbMlo12* in rubber tree and bioinformatic analysis. A 1,800-bp product was obtained by reverse transcription-polymerase chain reaction (RT-PCR) amplification with the leaf complementary DNA (cDNA) of rubber tree susceptible clone CATAS7-33-97. It contained a 1,725-bp open reading frame (ORF) encoding a putative protein of 574 amino acid residues (Fig. 1 and Supplementary Fig. S1). The genomic sequence of *HbMlo12* was

3,412 bp in length. Alignment of the genomic and cDNA sequences of *HbMlo12* in susceptible clone CATAS7-33-97 indicated that *HbMlo12* has 14 introns and 15 exons (Fig. 2). Blastp query in the *A. thaliana* proteome database showed that the putative protein shared the highest similarity with AtMlo12 (AT2G39200; with 63% identity). It was designated as *HbMlo12* (GenBank accession number KX499853). The predicted molecular mass of the deduced HbMlo12 protein was 65.89 kDa, with a pI of 8.98. Based on structural properties analyzed by the NCBI conserved domain search, HbMlo12 was a typical Mlo protein with a conserved domain of Mlo superfamily. Prediction by TOPCONS 2.0 indicated that HbMlo12 contains seven transmembrane domains (Fig. 1) (TM1 to TM7). The C-terminal D/E-F-S/T-F tetrapeptide sequence, which is one of several motifs characteristic of Mlos associated with powdery mildew susceptibility, is found in HbMlo12 (Fig. 1, asterisks). Furthermore, three distinctive regions identified in the Mlos associated with powdery mildew susceptibility are also present in HbMlo12 (motifs 25, 26, and 27) (Fig. 1, boxes). Taken together, these characteristics support HbMlo12 as a typical seven-transmembrane Mlo protein.

Multiple sequence alignment and phylogenetic analysis of the *HbMlo12* and other Mlo proteins from various species. Multiple sequence alignment showed that HbMlo12 shared high identity with Mlo proteins with known functions in

Fig. 6. Expression of *HbMlo12* **A**, in different tissues and **B**, in response to powdery mildew inoculation. **A**, Samples of different tissues were collected from 10-year-old healthy tapping trees of CATAS7-33-97. N.D. means not detected. **B**, Grafted budding seedlings of CATAS7-33-97 with tender leaves in the pale green period were used for powdery mildew inoculation. The final value of relative gene quantification was indicated as n -fold change in comparison with the untreated control (0 h). All gene expressions were performed for three biological replicates, and six plants were used in each independent trial. The data were analyzed by one-way analysis of variance and Duncan's multiple comparison tests. Data are showed as means $\pm$ standard errors. Bars with different lowercase letters show significant differences at the $P < 0.05$ level.

modulating antifungal defense, with 63, 60, 60, and 44% identity with *A. thaliana* AtMlo12, AtMlo2, AtMlo6, and barley HvMlo, respectively (Fig. 1). To reveal the relationship of HbMlo12 with other Mlo proteins, 120 Mlos, including *Arabidopsis* AtMlo1 to AtMlo15, *Cucumis sativus* CsMlo1 to CsMlo14, *Solanum lycopersicum* SIMlo1 to SIMlo17, *Vitis vinifera* VvMlo1 to VvMlo17, *Malus domestica* MdMlo1 to MdMlo21, barley HvMlo1 to HvMlo9, *Triticum aestivum* TaMlo1 to TaMlo7, *Oryza sativa* OsMlo1 to OsMlo12, and another 5 Mlos from rubber tree, as well as other Mlos with known functions in modulating antifungal defense were used for phylogenetic analysis (Supplementary Table S1). The result showed that these proteins could be divided into seven clades (I to VII) (Fig. 3). HbMlo12 and susceptible Mlo from dicot plant species, including AtMlo12, AtMlo6, AtMlo2, SIMlo6 (syn. SIMlo1), CsMlo5 (syn. CsaMlo8), MdMlo19, VvMlo3, VvMlo4, pepper CaMlo2, and pea PsMlo1, were grouped into clade V with 98% bootstrap support value (Fig. 3). However, clade IV included Mlo from monocot plant species, including barley HvMlo1 (syn. HvMlo) and wheat TaMlo1A, TaMlo1B, and TaMlo1D (Fig. 3). The high similarity of HbMlo12 with susceptible Mlo from dicot plant species suggested that HbMlo12 might be an Mlo candidate conferring susceptibility to powdery mildew in rubber tree.

HbMlo12 located in the endoplasmic reticulum membrane.

According the online analysis tool PSORT prediction (<https://psort.hgc.jp/form.html>), the most probable localization of HbMlo12 was

in the endoplasmic reticulum membrane, with probability value of 0.685 (Fig. 4A). TOPCONS 2.0 prediction server analysis revealed that there were seven transmembrane regions within HbMlo12 (Fig. 4B). To determine the subcellular localization of HbMlo12, the *Agrobacterium* strain GV3101 with the target gene expression cassette 35S:HbMlo12:monomeric green fluorescent protein (mGFP) was transformed into tobacco epidermal cells. The construct HbMlo12-fused mGFP was expressed under the control of the CaMV35S promoter. The transformed tobacco epidermal cells were observed for green fluorescent protein (GFP) signal using Leica TCS SP8 MP laser confocal fluorescence microscopy (Leica Microsystems Inc.). The control mGFP was observed throughout tobacco epidermal cells, whereas HbMlo12:GFP fusion protein was expressed in the endoplasmic reticulum membrane of tobacco epidermal cells (Fig. 5).

Expression analysis of HbMlo12 in rubber tree. Expression analysis in different tissues of tapping trees of CATAS7-33-97 showed that *HbMlo12* transcript exhibited tissue specificity. *HbMlo12* transcript was not detected in stem, latex, and bark. However, its expression levels were 24-, 248-, and 260-fold in root, leaf, and flower, respectively, while comparing that in the stem (Fig. 6A). This indicated that *HbMlo12* was preferentially expressed in the flower and leaf. Therefore, expression profile analyses of *HbMlo12* under different treatments were performed in the leaves of grafted budding seedlings of CATAS7-33-97.

Fig. 7. Expression profiles of *HbMlo12* after treatment with various phytohormones, H_2O_2 , wounding, and drought stress. Grafted budding seedlings of CATAS7-33-97 with fully expanded leaves in the dark green period were used for various treatments, including treated with 5 mM salicylic acid (SA), 3 mM gibberellic acid (GA), 200 μ M methyl jasmonate (MeJA), 1.0% (vol/vol) ethephon (ET), 200 μ M abscisic acid (ABA), 100 μ M indole-3-acetic acid (IAA), or 2% (vol/vol) H_2O_2 ; wounding; and drought stress by withholding irrigation. All gene expressions were performed for three biological replicates, and six plants were used in each independent trial. The *HbMlo12* expression in the tested samples was first calculated relative to that in control plants (seedlings treated with 0.05% [vol/vol] ethanol were used as the control for chemical treatment, the well-watered plants were used as the control for drought stress treatment, and the unwounded plants were used as the control for wounding treatment). The final value of relative gene quantification was indicated as *n*-fold change in comparison with the untreated control (0 h). The data were analyzed by one-way analysis of variance and Duncan's multiple comparison tests. Data are showed as means $\pm$ standard errors. Bars with different lowercase letters show significant differences at the $P < 0.05$ level.

Because phylogenetic analysis suggested that *HbMlo12* might be a candidate *Mlo* gene conferring susceptibility to powdery mildew in rubber tree, the transcriptional regulation of *HbMlo12* expression in response to powdery mildew infection, plant hormones, and abiotic stimuli was investigated intensively in this study. As shown in Figure 6B, *HbMlo12* expression level was significantly increased in response to powdery mildew infection, and the highest expression level occurred at 6 h after inoculation, which increased 4.5-fold compared with the untreated control (0 h). This result indicated that *HbMlo12* was involved in modulating antifungal defense.

Application of exogenous ET (releasing ethylene) caused a distinct increase in *HbMlo12* expression in the leaves of rubber tree, and the highest expression level occurred at 6 h after treatment, which increased 11-fold compared with that in the control (0 h) (Fig. 7). Additionally, *HbMlo12* transcript was quickly induced by salicylic acid (SA) treatment and reached the maximum at 0.5 h after treatment, which increased 2.2-fold compared with that of the untreated control (0 h) (Fig. 7). Interestingly, *HbMlo12* expression was also quickly induced by gibberellic acid (GA) treatment and reached the maximum level at 0.5 h after treatment, but it showed a trend of decrease from 2 to 24 h (Fig. 7). By contrast, the amount of *HbMlo12* transcript was reduced after methyl jasmonate (MeJA) treatment. The lowest expression level was detected at 24 h, reducing by 85% compared with that of the untreated control (0 h) (Fig. 7). *HbMlo12* expression was also downregulated by application of ABA and under drought stress, and the lowest expressions were present at 2 h and 5 days after treatments, respectively (Fig. 7). However, there was no significant change in *HbMlo12* expression after indole-3-acetic acid (IAA), H₂O₂, and wounding stimuli.

DISCUSSION

The *Mlo* gene family has gained importance owing to its ubiquitous presence in higher plants and their classification into the plant defense-related gene family (Deshmukh et al. 2016). *Mlo* members have been found and identified in different plant species. The functions of most *Mlo* proteins are well studied in model plants and important crop species, such as *Arabidopsis*, barley, rice, wheat, etc. However, few *Mlo* functions have been described in tree species. In this study, we isolated an *Mlo* gene from rubber tree, and homology analysis supported it as *Mlo12* of rubber tree with high similarity with *Arabidopsis AtMlo12* (Fig. 3). Topology of *HbMlo12*, like other susceptible *Mlos*, contains seven transmembrane domains (Fig. 4B). Furthermore, subcellular localization in tobacco epidermal cells indicated that *HbMlo12* was localized to endoplasmic reticulum membrane (Fig. 5), which was different from many *Mlo* proteins locating to the cytomembrane (Devoto et al. 1999).

HbMlo12 shared the highest similarity with *AtMlo12* in the *Arabidopsis* protein database, with 63% identity in their amino acid sequences. Interestingly, *HbMlo12* and dicot *Mlo* proteins with known functions in modulating antifungal defense (including *AtMlo2*, *AtMlo6*, *AtMlo12*, and *SIMlo1*) share a specific carboxy-terminal tetrapeptide motif (D/E-F-S/T-F) (Fig. 1), which is a motif characteristic of barley *Mlo* orthologs (Panstruga 2005; Reinstadler et al. 2010). Furthermore, three distinctive regions identified in the *Mlos* associated with powdery mildew susceptibility are also

present in *HbMlo12* (motifs 25, 26, and 27) (Fig. 1) (Rispaill and Rubiales 2016). Phylogenetic analysis showed that *HbMlo12* together with susceptible *Mlo* proteins from dicot plant species, including *AtMlo12*, *AtMlo6*, *AtMlo2*, tomato *SIMlo1*, pepper *CaMlo2*, and pea *PsMlo1*, were grouped into clade V with 98% bootstrap support value (Fig. 3). Clade IV included *Mlo* from monocot plant species, including barley *HvMlo*, wheat *TaMlo1A*, *TaMlo1B*, and *Mlo1D* (Fig. 3). This result is in accordance with previous studies (Acevedo-Garcia et al. 2014; Chen et al. 2014; Deshmukh et al. 2014; Jiwan et al. 2013; Konishi et al. 2010; Liu and Zhu 2008; Zhou et al. 2013). In grapevine, three members of the clade V (*VvMlo3*, *VvMlo4*, and *VvMlo17*) were found to be specifically induced at the transcriptional level during infection by grapevine powdery mildew (Feechan et al. 2008). However, only *VvMlo3* and *VvMlo4* partially rescue the *Atmlo2/6/12* triple mutant (Feechan et al. 2013). Phylogenetic clade V of apple *MdMlo11* and *MdMlo19* was upregulated on powdery mildew inoculation. The knockdown of *MdMlo19* induces a very significant level of resistance, whereas the knockdown of *MdMlo11*, alone or in combination with *MdMlo19*, did not result in any reduction or additional reduction of susceptibility compared with *MdMlo19* alone (Pessina et al. 2016). All *Mlo* proteins from dicot species that are known to be associated with powdery mildew susceptibility sort into clade V, and monocot *Mlo* proteins sort into clade IV. *Mlo* proteins involved in powdery mildew interaction seem to be restricted to these two clades (Acevedo-Garcia et al. 2014; Consonni et al. 2006; Elliott et al. 2002; Kim et al. 2002b; Wang et al. 2014).

In barley, induced and natural loss-of-function *HvMlo* alleles provide durable broad-spectrum powdery mildew resistance (Büschges et al. 1997; Piffanelli et al. 2004). When *A. thaliana Mlo* genes were challenged with powdery mildew, the mutant of *Atmlo2* showed no disease symptoms. However, the microscopic examination showed that resistance in *Atmlo2* was incomplete only when the *Atmlo2 Atmlo6 Atmlo12* all mutate and the *Atmlo2/6/12* triple mutant was fully resistant to the fungal pathogen (Consonni et al. 2006). Complementation experiments using virus-induced gene silencing assays in tomato suggested that loss of *SIMlo1* function is responsible for powdery mildew resistance (Bai et al. 2008). Silencing of *CaMlo2* in pepper plants enhances resistance against virulent *Xanthomonas campestris* pv. *vesicatoria* but not against avirulent *X. campestris* pv. *vesicatoria*. *CaMlo2* overexpression in the genus *Arabidopsis* enhances susceptibility to *Pseudomonas syringae* pv. *tomato* and *Hyaloperonospora arabidopsidis* (Kim and Hwang 2012). The loss of *PsMlo1* (also known as *Er1*) function confers durable broad-spectrum powdery mildew resistance in pea (Humphry et al. 2011). In apple, the natural truncated allele of *MdMlo19* is present in the T-1201 insertion of a single nucleotide, which caused the formation of an early stop codon and resulted in a truncated protein lacking 185 amino acids, including the calmodulin binding domain (Pessina et al. 2017). In bread wheat, artificial mutation created by knockout of all three *Mlo* alleles using the TALEN and CRISPR-Cas9 systems confers heritable broad-spectrum resistance to powdery mildew (Wang et al. 2014). Therefore, screening of natural mutation or artificial mutation created by knockout of susceptible *Mlos* is an effective way to breed crops with powdery mildew resistance.

TABLE 1. Sequences of the oligo nucleotides used in this study^a

Name	Sequence (5' to 3')	Name	Sequence (5' to 3')
HbMlo12-F	CTGCATCAAGAGGAAGCTTTACTAC	HbMlo12-R	CAATAACAAGGTTTAGCCTGAAAA
HbMlo12- <i>Xho</i> I-F	CCGCTCGAGATGTCTGATACTGGAA	HbMlo12- <i>Bam</i> HI-R	CGGGATCCATTCTTGAATGTGAAC
HbMlo12-QF	GTCTGCCTTGTGATGATTG	HbMlo12-QR	TGTCTTACTCTTGTGCTGAT
HbActin-F	GATGTGGATATCAGGAAGG	HbActin-R	CATACTGCTTGGAGCAAGA

^a The underlined sequence indicates the restriction enzyme cutting site.

Expression analysis of *Mlo* genes in various species reveals that each *Mlo* gene has a unique expression pattern and is regulated differently by a variety of biotic and/or abiotic stimuli (Chen et al. 2006; Kim et al. 2002a; Piffanelli et al. 2002). In this study, we found that the highest expression of *HbMlo12* was in the flower and leaf of rubber tree (Fig. 6A). Other *Mlos* in clade V, such as *AtMlo2*, *AtMlo6*, and *AtMlo12*, showed expression in vascular tissues of leaf. *AtMlo2* and *AtMlo12* genes also expressed in root vascular tissues (Chen et al. 2006). The research of Chen et al. (2006) showed that *AtMlo2*, *AtMlo6*, and *AtMlo12* have abundant expression in leaf and that they seem to be the most responsive *AtMlo* genes under biotic stresses. *HvMlo* transcript abundance increases in response to powdery mildew, rice blast, wounding, paraquat treatment, and a wheat powdery mildew-derived carbohydrate elicitor and also, during leaf senescence (Piffanelli et al. 2002). The drought stress treatment slightly induced *CaMlo2* transcription before 24 h and significantly induced in pepper plants by ABA (Lim and Lee 2014). Wounding induces the expressions of *AtMlo6* and *AtMlo12* (Chen et al. 2006). *CaMlo2* expression was induced in pepper leaves by *X. campestris* pv. *vesicatoria* infection (Kim and Hwang 2012). *HbMlo12* expression was significantly induced by powdery mildew infection (Fig. 6B). Moreover, *HbMlo12* transcript was quickly induced by SA and GA treatments and reached the maximum at 0.5 h after treatments. By contrast, *HbMlo12* transcript was downregulated by MeJA, ABA, and drought stress treatments. There was no significant change in *HbMlo12* expression after IAA, H₂O₂, and wounding stimuli. However, *HbMlo12* expression was dramatically induced by exogenous ET application, which was much more significant than when treated by other phytohormone (Fig. 7). SA, JA, and ET are key signaling molecules in regulating plant disease resistance (Dong 1998). SA is a necessary and sufficient signal for systemic acquired resistance (Gaffney et al. 1993). ET and JA mediate the activation of various defense responses and resistance to certain pathogens (Dong 1998; Pieterse and van Loon 1999). The induced expression of *HbMlo12* by powdery mildew and exogenous ET and SA applications in susceptible clone CATAS7-33-97 suggested that there was a putative role for *HbMlo12* in plant ET- and SA-dependent defense. However, additional molecular mechanisms of *HbMlo12* in response to powdery mildew infection and other pathogens infections require additional investigation. The results in this study are vital in understanding *Mlo* gene evolution and biological function in rubber tree and developing powdery mildew resistance varieties using reverse genetics.

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