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Genome-wide identification and analysis of the *MLO* gene families in three *Cucurbita* species

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Abstract: Powdery mildew (PM) is a major fungal disease in the *Cucurbita* species in the world, which can cause significant yield loss. The *Mildew Locus O* (*MLO*) family genes play important roles in the PM stress response. In this paper, twenty, twenty-one, and eighteen candidate *MLO* genes in *Cucurbita moschata*, *Cucurbita maxima* and *Cucurbita pepo,* respectively, were identified and designated as *CmoMLO*, *CmaMLO* and *CpeMLO,* respectively. The phylogenetic analysis indicated that these MLOs were divided into five clades and the number of MLOs belonging to clade V in the *Cucurbita* species was more than that in other crops. Furthermore, the expression analysis in the susceptibility (S) and resistance (R) lines showed that *CpeMLO1*, *CpeMLO2* and *CpeMLO5* might be involved in the susceptibility response. *CpeMLO4* and *CpeMLO6* showing opposite expression patterns in the R/S lines might be involved in the resistance response. All these data would be beneficial for future functional analysis of *MLO*s in the *Cucurbita* species.

Keywords: *Cucurbita maxima*; *Cucurbita moschata*; *Cucurbita pepo*; expression analysis; phylogenetic analysis; powdery mildew; resistance response; susceptibility response

Powdery mildew (PM) is a widespread plant disease, provoked by airborne spores of the ascomycete fungi. PM has a wide range of hosts and can attack over 9 000 dicot and over 65 monocot plants, including nearly all the Cucurbitaceous species causing significant harvest loss and quality decline (Inuma et al. 2007). *Podosphaera xanthii* (*P. xanthii*) is considered the dominant race of the PM pathogen in most countries (Sedlarova et al. 2009). *P. xanthii* has host specificity, for example, it can infect watermelons, but it cannot infect barley, while *formae speciales* (f.sp.) *hordei* (*Bgh*) are strictly specialised on barley (Vakalounakis et al. 2010; Zhu et al. 2010). The diversity and specialisation of the host and physiological races increase the complexity of the interaction mechanism.

The MLO family is important for the PM resistance (Berg et al. 2017). MLOs are plant polytopic membrane proteins shown to harbour seven transmembrane-spanning domains (TM) and a calmodulin-binding domain (CaMBD) (Iovieno et al. 2015). Unlike the Resistance gene (R-gene), some *MLO*s were identified as having PM susceptibility factors contributing to the infection and supporting compatibility with the pathogens, and some of the functional-lost *mlo* mutants (T-DNA insertion mutant, RNAi or VIGS inhibitory mutant) showed a broad-spectrum resistance (Van Schie & Takken 2014). The discovery of these *MLOs* called a susceptibility gene (S-gene) has always been a focus of attention for researchers. So far, all of the *MLO* S-genes belong to clade IV and V in monocots and dicots, respectively, and have played important roles in PM susceptibility (Appiano et al. 2015; Andolfo et al. 2019). For example, the function loss of *SlMLO1* and its two closely

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related homologs, *SlMLO5* and *SlMLO8*, resulted in a higher resistance than that associated with the *ol-2* mutation (Zheng et al. 2016). The over-expression of *CsaMLO11* in the tomato mutant partially restored the PM susceptibility and the over-expression of *CsaMLO1* or *CsaMLO8* completely restored the PM susceptibility (Berg et al. 2017). However, not all genes in clade IV and V play a significant role in PM susceptibility (Zheng et al. 2016). Furthermore, not all of them could completely inhibit the pathogen infection, such as *VvMLO11* and *VvMLO13* in *Vitis vinifera*, and *SlMLO1* in tomatoes (Pessina et al. 2016; Zheng et al. 2016).

Compared with other Cucurbitaceae crops, the *Cucurbita* species ($2n = 2\times = 40$) have a smaller genome size, more genetic variation and extensive resistance. Therefore, it is necessary to identify new S-genes and apply them to resistance breeding. In this paper, the Hidden Markov model (HMM) was used to search the Cucurbit Genomics Database, and the MLO homologs were identified by comparing with the MLO specific domain (PF03094). Finally, fifty-nine candidates were identified: twenty in *Cucurbita pepo*, twenty-one in *Cucurbita moschata*, and eighteen in *Cucurbita maxima* designated as *CmCmoMLO1-20*, *CmaMLO1-21*, and *CpeMLO1-18*, respectively. The numbers of MLOs varied in different *Cucurbita* species with a similar genomic background, which might be explained by the occurrence of a whole genome duplication (WGD) event (Montero-Pau et al. 2018). Detailed information on these sequences are listed in Table S1 in the Electronic Supplementary Material (ESM).

To study the phylogenetic distances of these fifty-nine MLOs, a phylogenetic tree was constructed using the UPGMA method of the MEGA 6.0 based on their protein sequences. Fifteen *Arabidopsis thaliana* MLOs (AtMLO) acquired from The Arabidopsis Information Resource (TAIR) database were used as a reference. As shown in Figure 1, seventy-four MLOs were divided into five clades. The MLOs from clade I were further divided into two sub-clades (Ia and Ib). Interestingly, all of the six MLOs containing the fungal_trans domain in the *Cucurbita* species were distributed in clade I. All the MLOs containing a signal peptide domain came from clade III. In clade V, twenty MLOs in the *Cucurbita* species were grouped with AtMLO02, AtMLO06 and AtMLO12. The MLOs

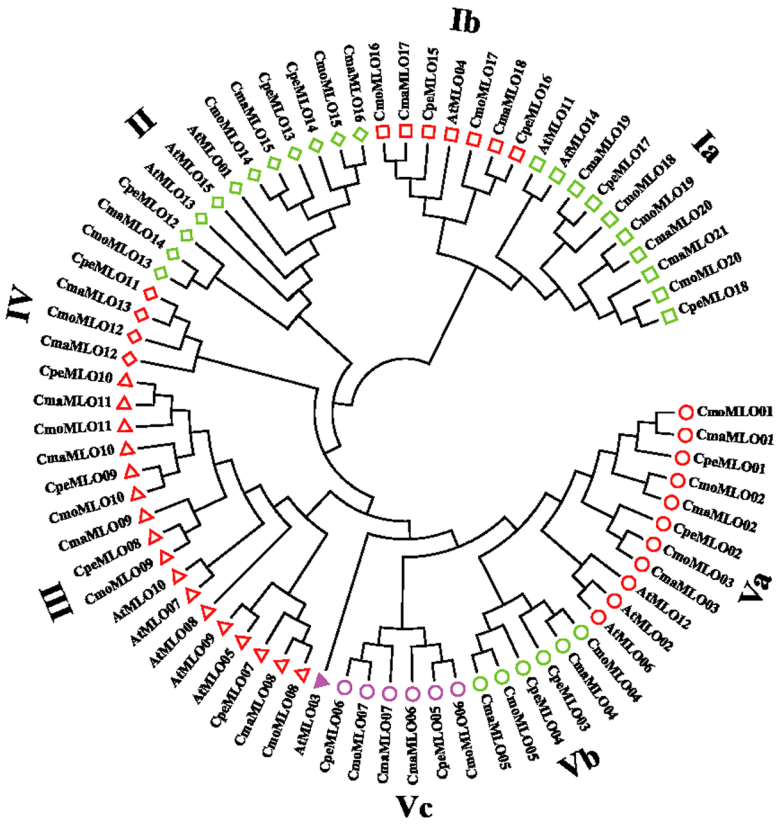

Figure 1. The molecular phylogenetic analysis of the *MLOs* in the Cucurbitaceae crops
The different groups are represented by different colours, and their names are shown on the outsides of the circle

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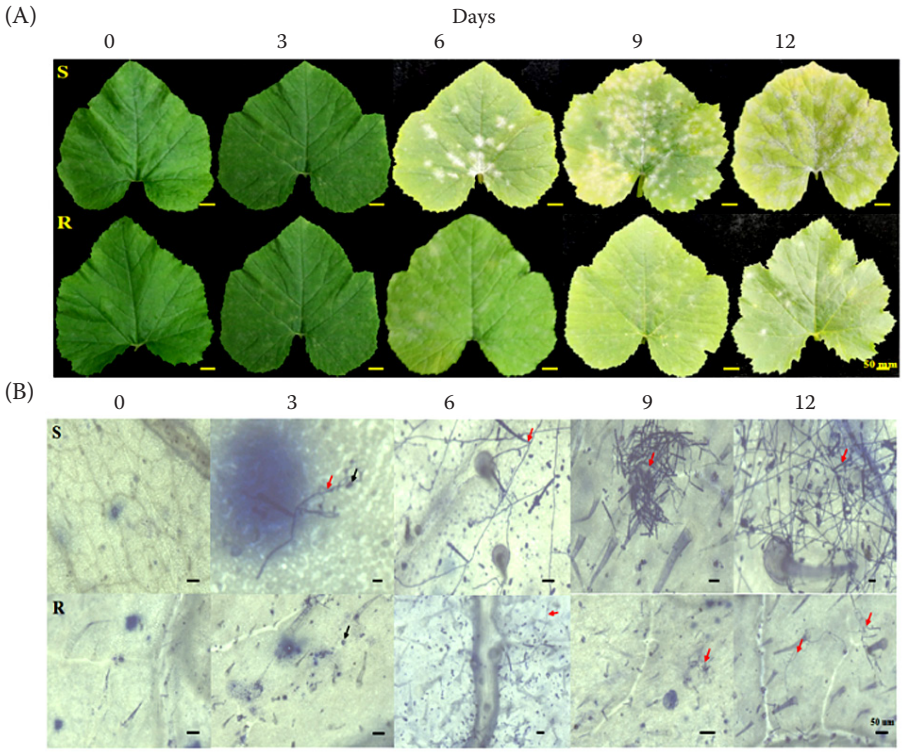

Figure 2. Symptom changes (A) and accumulation of fungi (B) in the *Cucurbita pepo* leaves under the Powdery mildew infection
The scale was marked with red and black lines; the red arrows indicate the fungal hyphae and the dark arrows indicate the conidium

from clade V were further divided into three subclades (Va, Vb and Vc). Interestingly, AtMLO03 fell into a separate category.

At the two-leaf stage, seedlings of the resistant *Cucurbita pepo* 379 line (V05B0381, R) and the susceptible *Cucurbita pepo* 341 line (V05B0348, S) introduced from the National Crop Germplasm Resource Platform of China were subjected to a *P. xanthii 2F* infection with a concentration 2×10^6 spores/mL. The control plants were mock-inoculated with distilled water. Samples were collected at 0, 3, 6, 9 and 12 days after inoculation, respectively. For the PM pathogen microscopic analysis, the second leaves of different treatments were decolourised in a mixture of acetic acid and ethanol (V : V = 75 : 25) at 70 °C for at least 30 min. After being cleaned with ddH_2O, they were stained with a mixture of methyl blue, lactic acid, glycerol and distilled water (W : V : V : V = 1 : 1 : 1 : 10) for 24 h. Finally, these leaves were stored in 50% glycerin and image observations were taken under a microscope (Nikon, SMZ18, Japan). No changes were observed in the mock treatment (data not shown). As shown in Figure 2A, there was an obvious PM accumulation on the leaf surface of the S-line at 12 days post infection (dpi), while less change was observed in that of the R-line at 12 dpi. The microscopic observations of the PM fungi showed that the germination speed of the conidium and hyphae accumulation was faster in the S-line than that in the R-line. The conidium seemed to germinate earlier by 3 days in the S-line (Figure 2B). These results suggested that the growth speed of the PM on the R-line was slower than that on the S-line.

To study roles of the *CpeMLO*s, their relative expression levels in both the R-line and S-line in response to the PM infection were analysed by a RT-qPCR (Figure 3). Non-coding regions were selected for the primers' design by online software (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool) (listed in Table S2 in ESM). The *RPL44* gene encoding the 60S ribosomal protein L44 was used as a control for normalisation. The RT-qPCR was performed with a Multicolor Real-Time PCR Detection System (CFX96; Bio-Rad) using an EvaGreen 2X qPCR MasterMix (MasterMix-S-XL; ABM). The relative gene expressions were calculated as described by Livak & Schmittgen (2001). The transcript levels of *CpeMLO01*, *02*, *05*, *12*, *15*, *16*, *17*, and *18* were all significantly increased in both lines under the PM infection. While the expression levels of *CpeMLO03*, *07*, and *14* were significantly depressed in both lines

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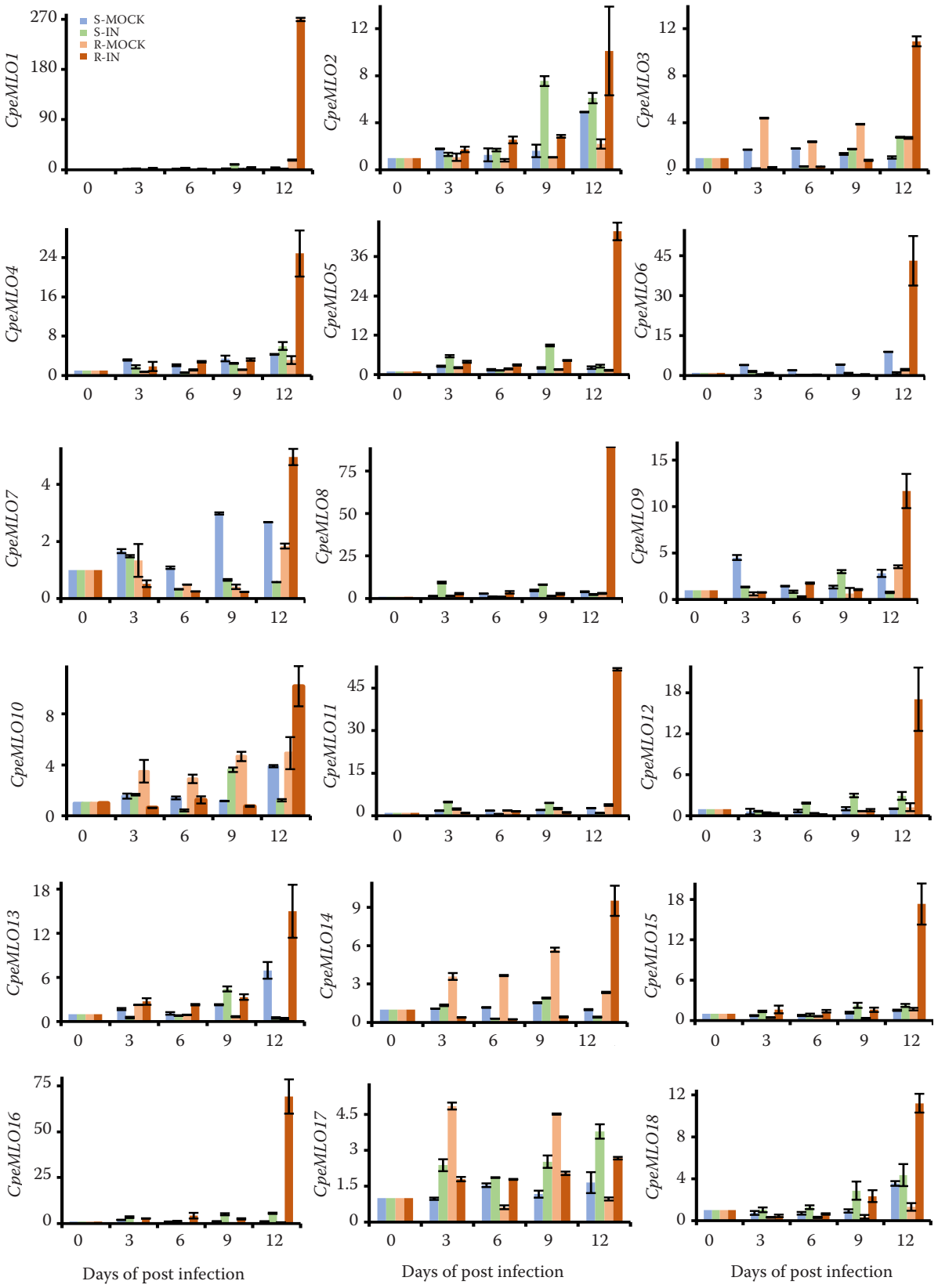

Figure 3. Expression analysis of *CpeMLO*s in the *C. pepo* leaves under the Powdery mildew infection by the RT-qPCR
The values represented as the mean ± standard deviation of three replicates; expression changes over two-fold were considered to be significant

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after infection except at 12 dpi. *CpeMLO04*, *06*, and *13* were remarkably suppressed in the S-line and up-regulated in the R-line after infection. *CpeMLO08*, *09*, *10*, and *11* in the S-line all exhibited irregular expression patterns, while *CpeMLO8* and *9* were increased in the R-line. Since the MLOs from clade V in the dicots played a major role in the PM susceptibility, the *MLO* genes functioning as susceptibility factors were strongly up-regulated under the PM infection (Seifi et al. 2014). In this study, only three *MLOs* (*CpeMLO01*, *02*, and *05*) in clade V were up-regulated, making them candidates to act as PM susceptibility genes. *CpeMLO04*, and *06* showing opposite expression patterns in the susceptibility or resistance lines might be involved in the resistance response, while a different number of candidates were identified in other plants (Consonni et al. 2006). This difference might be due to the different patterns of the genome expansion and evolution in the different species.

In conclusion, fifty-nine *MLOs* were identified in three *Cucurbita* species. The RT-qPCR analysis under the PM infection showed that the expression patterns of *CpeMLO01*, *02*, and *05* in clade V were inferred to be candidate PM susceptibility genes. *CpeMLO04* and *06* in clade V had the characteristics of the R-gene. Our analysis would benefit the further functional identification of MLOs in pumpkins and facilitate resistance breeding.

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