Analysis of Powdery Mildew Resistance in Wild Melon MLO Mutants

CHENG Honga,*, KONG Weipinga, Lü Junfenb, and LI Jipingc

a Institute of Vegetable, Gansu Academy of Agricultural Sciences, Lanzhou 730070, China
b College of Veterinary Medicine, Gansu Agricultural University, Lanzhou, 730070, China
c Institute of Plant Protection, Gansu Academy of Agricultural Sciences, Lanzhou 730070, China

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Abstract
Wild species have a potential value in crop breeding. Explore MLO gene which related with powdery mildew natural resistance is very important for improving the quality of melon. Resistance to powdery mildew was examined in cultivar and wild species by leaf inoculation. The wild germplasms showed resistance to powdery mildew Race1. Cloning and sequence analysis of the CmMLO2 gene identified an 85 bp difference between the wild and cultivated species. The CmMLO2 gene was expressed in the wild germplasm after fluorescence-labeled Agrobacterium-mediated transformation. A positive transgenic plant showed successful invasion by powdery mildew Race1. These results suggested that the wild species might have failed to encode the MLO protein, thereby resulting in the MLO-negative regulation of powdery mildew, which in turn resulted in the broad-spectrum resistance of the wild species to powdery mildew.

Keywords: melon; CmMLO2; mutation; powdery mildew

1. Introduction
China is a major producer of muskmelon (Liu, 2003). However, powdery mildew often infects muskmelon, thereby resulting in significant losses in its production (Cheng et al., 2006). Selection of a powdery mildew-resistant breed is an effective way of preventing and controlling melon powdery mildew, and the exploration and application of powdery mildew resistance genes may facilitate in breeding disease-resistant cultivars.

MLO is a specific gene family in plants (Panstruga, 2005a). Researches have found that a recessive mutation in the MLO gene of barley shows resistance to a broad spectrum of powdery mildews, and the mlo mutant has been widely used as a resource for the breeding of wild barley in Europe (Lyngkjaer et al., 2000). Cheng et al. (2009) cloned 3 MLO genes from muskmelon, which are respectively named CmMLO1, CmMLO2, and CmMLO3. Expression analysis has shown that these genes are tissue-specific. CmMLO1 is mainly expressed in the cotyledon and flower and CmMLO2 is mostly expressed in euphylla (Cheng et al., 2012, 2013b); CmMLO3 is exclusively expressed in the fruitlet, root, and flower. CmMLO2 has a relatively close genetic and evolutionary relationship with AtMLO2, AtMLO6, and AtMLO12 in Arabidopsis. Bioinformatics analysis has indicated that CmMLO2 is the typical MLO gene that encodes 7 transmembrane (TM) proteins. Through induction of powdery mildew, the relative transcript level of CmMLO2 in the blade has been determined to be significantly higher than that of the other two homologous genes.

* Corresponding author. Tel.: +86 931 7754993.
E-mail address: chengjn@yeah.net

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Except for barley (Jørgensen, 1992), Arabidopsis (Devoto et al., 1999), and muskmelon, MLO gene has been detected in tomato (Bai et al., 2008), monthly rose (Kaufmann et al., 2012), rice (Elliott et al., 2002), and soybean (Shen et al., 2012). Interference and prevention of MLO expression by using antonymy, a new germplasm, has been generated through mutagenesis, and naturally mutated material all have resistance to a broad spectrum of powdery mildew (Panstruga, 2005b). During the selection of disease resistance of germplasm of wild muskmelon, we determined that it was resistant to powdery mildew. To further confirm whether the observed resistance is related to the MLO gene, artificial inoculation was adopted to study the resistance reaction of the wild material and cultivated variety against the powdery mildew. By comparing sequence differences in the CmMLO2 gene of the two cultivars, a CmMLO2-GFP transformation vector with fusion gene was established for the analysis of the functions of wild mutant to provide information for breeding muskmelon cultivars that were resistant to powdery mildew.

2. Materials and methods

2.1. Material cultivation and treatment

Test material G24 is a cultivated variety of susceptible muskmelon (Cucumis melo L.), and C18 is disease-resistant wild species (C. chamaejasme), both belonging to an advanced selfing line. These cultivars were sown in a plastic nutrition pot after accelerated germination, and then placed in a box under artificial climate, with a night temperature of 18 °C and a day temperature of 25 °C. When the seedlings reached the four-leaf stage, the powdery mildew germ Race1 was collected using a single loric (Podosphaera xanthii), which is widely distributed in the Gansu Province. The seedlings were then infected with the plant pathogen in a bioclean room by using the leaf inoculation method (Liu et al., 2010). Three days after inoculation, spots of powdery mildew on the blades of the seedlings were collected and used for RNA extraction.

2.2. Cytological observation of blades infected with powdery mildew

By reference to the method used by Yang et al. (2013), the blade was flicked with a rubber pipette bulb and then cut into small pieces of about 5 mm × 5 mm. These were then fixed in 2.5% glutaraldehyde dissolved in 0.1 mol·L⁻¹ phosphate buffer (pH 7.2); we then vacuumed the blades at room temperature until the material sank. The blade samples were fixed at 4 °C for 24 h, and then washed with 0.1 mol·L⁻¹ phosphate buffer for 15 min, and fixed in 1% osmic acid for 4 h. Ethanol gradient dehydration and transition with acetone were then performed. The blade samples were dried in a K-850 and coated by using a Hitachi E-1010 ion sputtering equipment. The samples were then examined under a TESCAN5136 scanning electron microscope (SEM) and imaged. The accelerating voltage was 20 kV.

2.3. Gene cloning and sequence analysis

Total RNA was extracted by using the TRIzol reagent (Beijing Tiangen), following the manufacturer’s instructions. cDNA was synthesized by using a Smart™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s recommendations. Using the published gene sequence of the MLO gene, Premier 5.0 software was used to design the following PCR primers. MloF: 5'-GCAAGCAATTGGAACCTCTAG-3', MloR: 5'-GCACGAGCCTGACCGCTAG-3'. The first strand of the generated cDNA was then used as a template to obtain the overall sequence of the MLO gene via amplification. The reaction conditions were as follows: pre-denaturation at 94 °C for 3 min; denaturation at 94 °C for 30 s, renaturation at 56 °C for 30 s, and extension at 72 °C for 50 s for a total of 25 cycles; and a final extension at 72 °C for 10 min. 1.2% Agarose gel electrophoresis (AGE) was performed to separate the PCR products, and purified by using a spin-column Sepharose gel (Sangon Biotech). The purified products were then inserted into a PGEM-T easy vector (Promega), and transformed into Escherichia coli TOP10 cells. Blue-white selection of bacterial colonies on a plate containing X-gal and IPTG was then performed. White bacterial colonies were then selected and further cultivated, which was followed by plasmid extraction. The isolated plasmids were then subjected to DNA digestion and PCR identification, of which positive and monoclonal PCR products were selected and sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing. Comparative sequence analysis of the two cultivars was then performed using the DNAMAN software.

2.4. Establishment of transformation vectors with fluorescence labeling

The transformation vector of CmMLO2 was constructed with pROK2 and pjit163GFP. First, primers to amplify the coding region of the CmMLO2 were designed, and restriction sites of BamHI and SalI were then added. To form a fused fluorescent protein, the termination codon TGA was mutated into GGA. CmMLO2-F: 5'-TATGAGTCCATGGCTGATTTGAAAGCTCCCTTAGG-3', CmMLO2-R: 5'-CGTTCGACTCTTCTTGAGCAAGAG-3'. Then, the plasmid pjit163GFP was used as a template to amplify the coding region of the GFP, and the restriction sites of SalI and KpnI were added. GFP-F: 5'-TAGGATCATGGTGAGCAAGGCGAGG-3', GFP-R: 5'-GGCTACCTTACTTTTGTACAGCTCGTCC-3'. Finally, the CmMLO2 and GFP segments were PCR amplified, and inserted into the pGEM-T easy vector. After verification through sequencing, the plasmids were extracted and subjected
restriction enzyme digestion by \textit{Sal} I. T4 ligase was added and incubated overnight, and again connected to the pGEM-T easy vector. After double digestion using \textit{Bam}H I and \textit{Kpn} I, the pROK2 plasmid was ligated by digestion with \textit{Bam}H and \textit{Kpn} I.

2.5. Genetic transformation and resistance identification of mutant C18

By \textit{Agrobacterium tumefaciens}-mediated leaf disc transformation of wild muskmelon, the fusion expression vector pROK-CmMlo2-GFP was transformed into mutant C18. Resistance selection on a culture medium supplemented with kanamycin at a concentration of 50 mg·L$^{-1}$ was performed to obtain calli, and a few tissues were selected for secondary screening under a fluorescence microscope. Positive tissues were selected for regeneration, and the greenhouse after breeding. Seeds that underwent selfing and pollination were selected and bred in manual climatic box. Then, resistance to powdery mildew was evaluated in seedling stage.

3. Results

3.1. Resistance of wild muskmelon germplasm C18 and cultivated variety G24 to powdery mildew

By using the leaf inoculation method, the cultivated variety G24 and wild germplasm C18 were inoculated with powdery mildew Race1 and incubated for 72 h. After incubation, a layer of powdery mildew was clearly observed at the site of inoculation on the blades of the G24 plants, and the scab rapidly spread after 5 d. On the other hand, no layer of powdery mildew was observed on the blade surface of the C18 wild cultivar (Fig. 1). Cultivation was continued in the climatic box, and no scab of powdery mildew was observed after 7 d, indicating that the wild germplasm C18 was resistant to the Race 1.

SEM observation showed tiny pubescences on the blade surface (Fig. 2, A). After inoculation, the spores of powdery mildew started to germinate (Fig. 2, B), which eventually resulted in the appearance of a germ tube (Fig. 2, C). The surface of the blade showed wrinkling, and dense net-shaped hypha were distributed across the entire blade (Fig. 2, D). With progression of the disease, the hypha of powdery mildew continued to spread across the blade, and tiny pubescences were detected on the surface of blades and were twined by hypha. Branches of capillitium stretched out from the columella, thereby forming a net. A long, cylindrically shaped conidiophore emerged from the hypha and stands straightly to separate branches. The oval metrocyte of spore outwards segments to form conidium in bunches, which gradually matured and detached (Fig. 2, E). The blade was longitudinally cut, which showed that the cell tissues were mostly normal. As the disease progressed, the space between epidermal cells gets clear, and the hypha was attached to the surface of epidermis cell, and then inserted into the epidermal cells of muskmelon, with its haustorium of monoplast used to absorb nutrients (Fig. 2, F). The blade surface and glandular hairs on the surface of the wild germplasm C18 were plump-eared and smooth (Fig. 2, G and H). The epidermis cells were compact and well aligned. No pathogenic hypha and conidium were observed on the blade. No significant hypha and spore were detected between cell tissues on the vertical section of the blade, and instead starch grains were observed (Fig. 2, I).

3.2. Sequence analysis of the MLO gene of the wild germplasm and cultivated variety

Sequence analysis indicated that the full length of the \textit{CmMLO2} gene of the G24 cultivar was 1713 bp in length and encoded a 570 amino acid protein that comprised 7 transmembrane helices, which is consistent with the sequence of GenBank Accession Number FJ713542. The full length of the \textit{CmMLO2_1} gene of C18 was 1798 bp in length. The two sequences were compared by using DNAMAN (Fig. 3), which showed that the middle part of C18 harbored 85 more basic groups than that in G24 (TGCAAGCAGGTTGAT GTCAATTTCTCAGAGGAAAATATATTTGTTGAA ACAAATAACGTTGTTAAATATAGTACC).
Fig. 2  SEM observation of cultivar G24 and wild species C18 after inoculation with powdery mildew
A–F: G24 (A. No inoculation; B. Spores germinated; C. Spores tube; D. Hyphae and spores; E. Conidiophore and mycelium; F. Longitudinal leaf);
G–I: C18 (G. Leaf surface; H. Leaf puberulent; I. Longitudinal leaf).

The positions of the signal peptide and cleavage site were predicted by using Signal 4.0 signal peptide prediction software, which is based on the neural network method (Fig. 4). The signal peptide included one region that was positively charged, one hydrophobic region, and one region with polarity and without an electric charge. The red line in Fig. 4 C-score indicates the score of the cutting position, green line S-score refers to the score of the signal peptide upstream of the cutting position, and the blue line Y-score the decided position of the signal peptide after considering both C-score and S-score. The appearance of the red, green, and blue lines near the peak value indicates the existence of the signal peptide. The arrow of G24 in Fig. 4 indicates the possible positions of the signal peptide. No features of the signal peptide were observed in C18, which shows that the wild germplasm C18 contained no correctly synthesized CmMLO2 protein.
3.3. Disease resistance of genetically transformed plants of the wild mutant

After establishing the pROK2-CmMlo2-GFP, this was transformed into the wild mutant C18 by using the Agrobacterium mediated method, and cultivated on the MS culture medium that contained kanamycin to select kanamycin-resistant callus tissues. After continuous cultivation, small calli were selected for secondary microscopic examination under a fluorescence microscope. The positive calli showed clear fluorescent signs (Fig. 5, A and B), whereas those without fluorescent signs were designated as negative calli (Fig. 5, C and D). After induced differentiation of calli and induction for rooting, the genetically transformed plant F₀ generation of the mutant G18 was generated. Fig. 5 indicates the resistance of F₁ generation after being inoculated with powdery mildew. Scabs of the powdery mildew were observed in positively transformed plants (Fig. 5, E), indicating that these were susceptible to disease. On the other hand, no scabs of the powdery mildew were detected in negative plants (Fig. 5, F), which was indicative of disease resistance.

Fig. 5  Fluorescence screening of callus on mutant C18 and identification of resistance to powdery mildew
A. Positive callus; B. Positive callus under fluorescence optics; C. Untransformed callus; D. Negative callus under fluorescence optics; E. Positive plant inoculated with powdery mildew and incubated for 5 d; F. Negative plant inoculated with powdery mildew and incubated for 5 d.
4. Discussion

In the present study, differences in resistance to powdery mildew between the cultivated variety and wild germplasm were compared through artificial inoculation of powdery mildew. The ultrastructure relationship between powdery mildew and the blade of the muskmelon is primarily studied through scanning electron microscopy, and the growth of hypha of germs of powdery mildew on the blade surface is discussed. During the process of invasion of the susceptible materials, no powdery mildew was observed at the wound of stoma or tiny pubescence. Evaluation of the longitudinal section indicated no mycelium invades in the host (muskmelon), although it had inserted into the epidermal cells of the muskmelon withhaustorium of the monoplast to absorb nutrients. Zhao (2003) showed that the appearance ofhaustorium of pathogenic bacteria causing powdery mildew induced the formation of new chloroplasts in the mesophyll cell of barley, and postponed the aging process of chloroplasts, thereby providing enough nutrition for the growth of the haustorium of pathogenic bacteria. Therefore, the infection mode and extended mode of germs of muskmelon were similar to those of graminaceous crops.

Xu et al. (2014) performed sequence analysis of the MLO gene family, which identified a total of 14 MLO gene family members in muskmelon. The size of the CmMLO2 gene (FJ713542) of the cultivated variety G24 was completely similar to that of MLO03C012438P1; both were 1,713 bp in length and encoded a 570 amino acid protein. Phylogenetic analysis indicated that MLO03C012438P1 was closely related to Arabidopsis AtMLO2, AtMLO6, and AtMLO12. The findings of the present study are also in agreement with that of a previous study (Cheng, 2009). In the 14 gene family members of the MLO gene of muskmelon, no homologous genes with a length of 1,798 bp were detected. Taken together, CmMLO2_1 cloned in the wild germplasm C18 is the mutant of the CmMLO2 gene.

The author has obtained the gene CmMLO2 related to powdery mildew from muskmelon by using RT-PCR, and established the expression vector of RNAi (pFGC1008-CmMLO2). Then, the cultivated muskmelon was transformed by leaf disc transformation mediated by A. tumefaciens, proving that the CmMLO2 gene with endogenesis knocked out by ihpRNAi can obtain muskmelon material that was resistant to powdery mildew (Cheng et al., 2013a). The MLO gene thus plays a very important role in the invasion process of powdery mildew in muskmelon, and this, to some extent, is equivalent to the susceptibility gene in the muskmelon.

The normal transcription and expression of the MLO protein are necessary for the successful invasion of powdery mildew (Freialdenhoven et al., 1996; Lyngkjaer et al., 2000; Zellerhoff et al., 2010). By inducing mutations, a change of coding sequence of MLO can be changed to induce plant resistance to powdery mildew (Bueschges et al., 1997; Panstruga, 2005a). In the 15 MLO family members of Arabidopsis, mutants AtMLO2, AtMLO6, and AtMLO12 can make mouseearcress become completely resistant to powdery mildew (Golovinomyces cichoracearum and G. orontii) (Consonni et al., 2006). Additional research results have proven that the deficiency of genetic transcription or mutation of the encoded cDNA sequence can result in the change of sequence of polypeptide, which will then cause the transformation of resistance against powdery mildew. In a research involving the wild tomato R26 strain against powdery mildew (Bai et al., 2008), the cDNA sequence of SlMlo1 showed the loss of 19 basic groups, which in turn causes a shift in the SlMlo1 in coding region. This may then cause premature termination of translation and lead to resistance to powdery mildew in tomato R26. The upregulation of MLO may prevent necrosis of mesophyllic cells, as well as provide enough nutrition for the development of pathogenic bacteria, which results in further invasion of other plant tissues. Sequence analysis of the MLO gene of the two cultivars and disease resistance identification that the mutation of CmMLO2_1 of the wild germplasm C18 may be related to natural resistance. The analysis of the signal peptide also proves that the mutated CmMLO_1 failed to normally encode the CmMLO2 protein. To verify that the resistance of the mutant to powdery mildew is caused by a mutation in the CmMLO2 gene, this was expressed in the mutated plant by establishing a genetically transformed expression vector that was tagged to a fluorescence label. This transformation experiment indicates that the mutation of the gene CmMLO2 is possibly related to the resistance of the wild germplasm against powdery mildew. The mutated strain of the MLO gene of the wild germplasm facilitates future research studies on the functions of genes related to melon powdery mildew. The wild strain C18 is a naturally mutated material with a stable condition and may be used in resistance breeding of muskmelon.

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References


