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Analysis of Powdery Mildew Resistance in Wild Melon *MLO* Mutants

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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*. Bio-informatics 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.

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Except for barley (Jφrgensen, 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. chate*), both belonging to an advanced selfing line. These cultivars were sown in a plastic nutrition pot after accelerated germination, and then place in a box under artificial climate, with a night temperature of 18 ℃ and a day temperature of 25 ℃. When the seedlings reached the four-leaf stage, the powdery mildew germ Race1 was collected using a single lorica (*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 ℃ 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^{TM} 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′-GCAAGCAGTGGTATCA ACGCAGAG-3′, MloR: 5′-GTATTTGCTGCTGCCCTGTACA TGA-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 ℃ for 3 min; denaturation at 94 ℃ for 30 s, renaturation at 56 ℃ for 30 s, and extension at 72 ℃ for 50 s for a total of 25 cycles; and a final extension at 72 ℃ 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 panel 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 *Bam*HⅠ and *Sal*Ⅰ were then added. To form a fused fluorescent protein, the termination codon TGA was mutated into GGA. CmMLO2-F: 5′-TAGGATCCATGGCTGAATGTG GAACAG-3′, CmMLO2-R: 5′-CTGTCGACTCCTTTGGCAA ATGAGAAG-3′. Then, the plasmid pjit163GFP was used as a template to amplify the coding region of the GFP, and the restriction sites of *Sal*Ⅰ and *Kpn*Ⅰ were added. GFP-F: 5′-TAGTCGACATGGTGAGCAAGGGCGAGG-3′, GFP-R: 5′-GCGGTACCTTACTTGTACAGCTCGTCC-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