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CONSTRUCTION OF EXPRESSION VECTORS OF THE MELON RESISTANCE GENE *FOM-2* AND GENETIC TRANSFORMATION

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Abstract

In this study, we constructed positive and negative expression vectors using the Melon Wilt Resistance Related Gene *Fom-2* and transformed the vectors into melon via the pollen tube pathway. In the field, 52 Kan-resistant M4-45 plants and 84 Kan-resistant MR-1 plants were transformed with the Antisense Vector, and the conversion rates were 3.28% and 4.26%, respectively. The PCR results showed that antisense vectors were integrated into the genome of *Cucumis melo*. The results of a special inoculation test of *Fusarium oxysporum melonis* showed that the incidence of Fusarium wilt in transgenic plants was 84% and that in non-transgenic plants was 96%. The contents of chitinase, polyphenol oxidase and peroxidase increased with the bacterial treatment time in transgenic plants and non-transgenic plants, and the activities of these three enzymes in transgenic plants were higher than those in non-transgenic plants.

Key words: Melon; *Fom-2;* Expression vector; Genetic transformation; Disease resistance identification.

Introduction

Cucumis melo (L.) is one of the most important horticultural plants and is one of the ten most recognized healthy fruits in the world. Wilt (Fusarium wilt) is a soil borne fungal disease that devastates cucurbits and causes yield loss worldwide (Mas *et al*., 1981; Sherf & MacNab, 1986; Zitter, 1999) and is caused by *Fusarium oxysporum* muskmelon biotypes (*Fusarium oxysporum* Schlecht f. sp. *melonis (F.o.m)* W.C. Snyder & H.N. Hans) that were discovered by (Leach & Currence, 1938). With increased production, the expansion of cultivated areas and the diversification of cultivation methods, the occurrence of this disease has also gradually increased.

Breeding for resistance to Fusarium wilt has become one of the most important targets in melon breeding. Introducing genetic resistance into cultivars is an effective means of protecting plants from disease (Cai *et al.*, 2009). Introducing exogenous genes via genetic engineering, creating new disease-resistant melon germplasm, and resolving the contradiction of the lack of disease-resistance resources have finally resulted in the breeding of new varieties of disease-resistant melon as the basic measure to control wilt disease. Currently, four specific physiological types of muskmelon wilt disease have been identified (0, 1, 2 and 1.2) (Risser *et al*., 1976), and three Fusarium wilt resistance genes have been discovered: Fom-1 gene (resistance to races 0 and 2 but not to 1), Fom-2 gene (anti-physiological small 0 and 1 but not 2) (Risser *et al*., 1976), and Fom-3 gene (resistance to races 0 and 2 but not to 1) (Zink & Gubler, 1985).

Many local and international scholars have separated and cloned muskmelon Fusarium wilt resistance genes by molecular marker technology, resulting in the isolation of *Fom-2* and *Fom-1* molecular markers and the cloning of the two genes (Luo *et al*., Garcia-Mas *et al*., 2001; Joobeur *et al.*, 2004; Brotman *et al*., 2013; Van Leeuwen *et al*., 2005; Oumouloud *et al*., 2008; 2009; 2012; 2013; 2015. Tezuka *et al*., 2009; 2011). *Fom-2* and *Fom-1* have been widely used in melon breeding and introduced into popularized varieties (Joobeur *et al*., 2004, Brotman *et al.*, 2013, Oumouloud *et al*., 2013).

In this study, to further clarify the *Fom-2* gene resistance to Fusarium wilt in muskmelon, the methods of construction of a *Fom-2* gene antisense expression vector, genetic transformation via the pollen tube pathway, and PCR identification and field resistance identification of transgenic muskmelon plants were used. The results will contribute to the development and utilization of resources to increase muskmelon resistance to Fusarium wilt and provide a foundation for molecular breeding.

Materials and Methods

Plant materials and strains: MR-1 for Muskmelon Fusarium Wilt Resistance of muskmelon M4-45 materials and *Fusarium oxysporum* muskmelon materials were provided by Northeast Agricultural University West Muskmelon Molecular Breeding Laboratory. The pMD18-T-*Fom-2* plasmid containing the target gene fragment of *Fom-2* was also obtained from the laboratory for construction and preservation. *Fusarium oxysporum* (type 1) was purified and preserved by this laboratory.

Primer design and target gene amplification: The full-length *Fom-2* and fragment primers according to the muskmelon *Fom-2* gene were cloned into the whole sequence (GenBank sequence number is AY58385) to design primers with primer software design (version 5), which were synthesized by the Shanghai Biological Engineering Company (Table 1)

Fom-2 full-length amplification: a 25 uL system: 2.5 μL of TransTaq Buffer, 2 μL of 2.5 mM dNTPs, 0.5 μL of HiFi Polymerase, 0.5 μL of 3' and 5' primers, 1 μL of cDNA, and 18 uL of water. Procedures: denatured for 3

min at 95°C, denatured for 1 min at 95°C, annealed for 1 min at 55°C, extended for 2 min at 72°C, programmed for 35 cycles, and extended for 10 min at 72°C after the reaction.

Fom-2 fragment amplification: a 25 uL system: 2.5 µL of TransTaq Buffer, 1.5 µL of 2.5 mM dNTPs, 0.4 µL of Taq Polymerase, 0.5 µL of 3' and 5' primers, 1 µL of cDNA, and 18.6 µL of water. Procedures: denatured for 3 min at 94°C, denatured for 1 min at 94°C, annealed for 30 s at 55°C, extended for 1 min at 72°C, programmed for 30 cycles, and extended for 7 min at 72°C after the reaction.

Construction of a positive and negative expression vector of the Fom-2 gene: The two restriction sites of X Ba I and BamH I were introduced into the full-length gene specific primer 5 'end using plasmid pMD18-T containing the Fom-2 gene as the template. The full-length Fom-2 gene fragment for amplification of the full-length gene was cloned into vector pMD19-T Simple at 37°C, followed by double enzyme digestion and 1% agarose gel electrophoresis for the detection of the recovered enzyme digestion product. The resulting 3222 bp DNA fragment was used to construct a sense expression vector.

PMD18-T BamH I and X Ba vectors of two enzymes of the muskmelon Fom-2 gene were digested, and 1% agarose gel electrophoresis was used to detect the enzyme digestion products. The recovered 580 bp gene fragments were used to construct the antisense expression vector.

The pBI121 expression vector was digested, and the recovered expression vector fragment was equivalent in size to the Fom-2 fragment formed by overnight ligation with T-ligase 4 at 16°C. The recombinant plasmid was inserted into the Fom-2 fragment reverse 35S promoter, TNOS. The ligation product was transformed into *Escherichia coli* DH5a. A tablet containing antibiotics was used to select a single colony. The bacterial suspension was shaken, followed by PCR and double enzyme digestion; then, the PCR product was verified by sequencing using the vector pS-Fom-2 and Antisense Vector pA-Fom-2 (Figs. 1 and 2)..

Table 1. Primers for full-length and fragment amplification of the Fom-2 gene

Gene	Sequence（5'-3'）
*Fom-2*XbaI-fwd	GCGGTCTAGAGTCAAGTGGGGTTGGAGC
*Fom-2*BamHI-rev	GCGGGGATCCCATTTTGCTTTGAAGAGT
Fom-2-fwd	TTGGCTTCCTCTTTACTT
Fom-2-rev	GTCTATTGTTTCGCTTCA

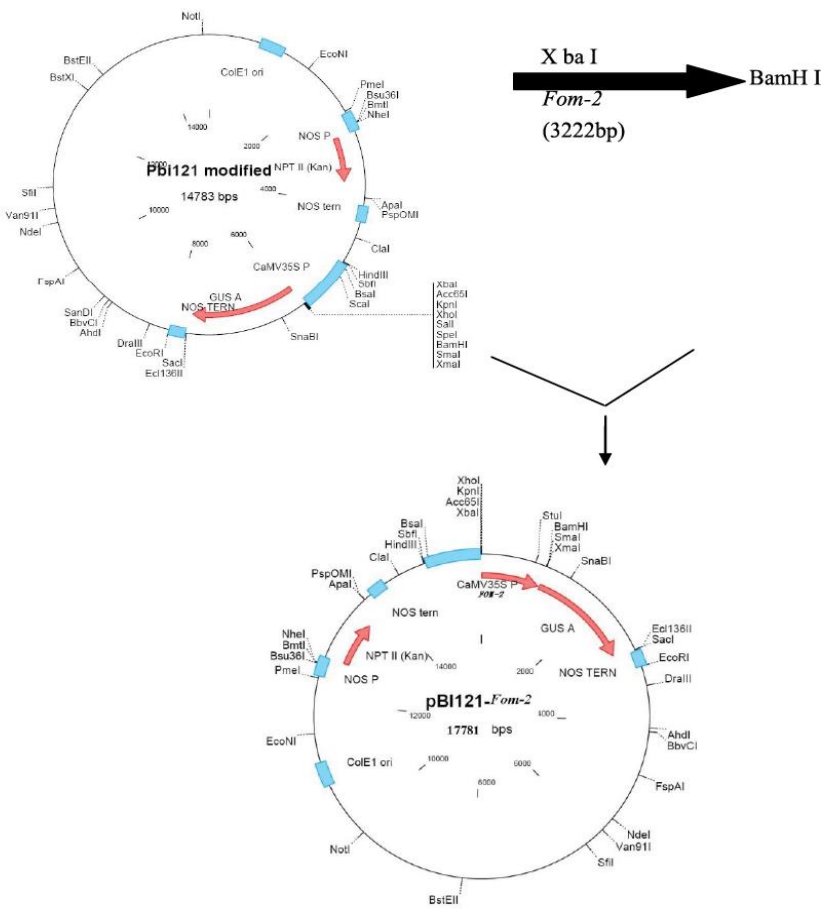


Fig. 1. Construction process of the *Fom-2* gene expression vector.

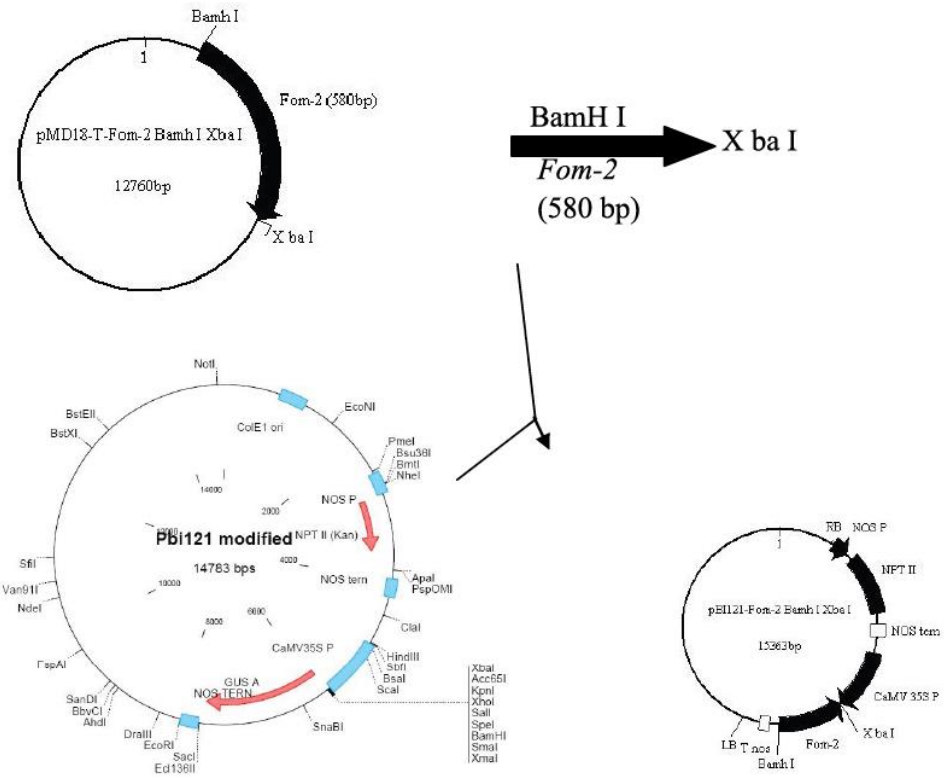


Fig. 2. Construction process of antisense expression vector of *Fom-2*.

Import solution preparation: In super clear Taichung, the *Escherichia coli* DH5 alpha, which contained the correct Fom-2 gene and antisense recombinant plasmid, was inoculated into LB liquid medium containing the corresponding antibiotics. The shaking table was heated at 37°C overnight at 170 rpm. The next day, the cells were collected and plasmids were extracted and detected by 1% agarose gel electrophoresis.

The concentration of the recombinant pA-Fom-2 plasmid in 1 L of the NDI000 trace solution was measured by UV spectrophotometry. The concentration of exogenous DNA was diluted to 750-1000 $\mu g.mL^{-1}$, and the remaining volume was stored at -20°C.

Introduction of exogenous gene: Tagging occurred at 9 a.m. on the morning of muskmelon pollination, and ovary injection was conducted at 9 p.m. the next day. The micro-syringe was disinfected with 70% alcohol, and the exogenous DNA solution was drawn into the syringe at a needle depth of 3-4 mm in 2 L. The plasmids were injected into the ovary, and an empty vector plasmid DNA solution was also injected. T0 seeds were obtained when the melon was ripe and harvested.

Screening of resistant plants by kanamycin: Many T0 seeds are harvested using the pollen tube pathway, and relying solely on molecular detection is time-consuming and costly. When a gene expression vector is constructed, the insertion of a genetic marker gene can effectively assist the molecular detection of the T0 transformed seeds. Among the most widely used genes is NPT-II. Plant cells that convert the NPT-II gene produce greater resistance to kanamycin (Kan), and the plant can grow normally. Kan is a commonly used marker for transgenic screening. At present, Kan has been successfully applied in the early screening and field detection of wheat and transgenic cotton, and several molecular tests verify the reliability of Kan detection. However, different plants have varying levels of resistance to Kan, and the same plant can have different levels of Kan sensitivity during different growth periods. Therefore, before determining the response of Kan-sensitive transgenic plants for muskmelon, non-transgenic plants were explored by measuring their resistance and yellowing index (chlorosis degree classification method based on Zhu Jianbo 2000), and ultimately, the optimum selection of muskmelon M4-45 concentration was determined to be 180 $mg.L^{-1}$, and the optimum concentration for muskmelon MR-1 screening was 170 $mg.L^{-1}$. The T0 seeds of melon M4-45 and melon MR-1 harvested by the pollen tube pathway were seeded into the seedling nursery. When the cotyledon was extended, the T0 generation was screened with the optimum concentrations indicated above.

PCR identification of resistant plants: The resistant plants were screened and cultured until seedlings grew 4-5 leaves. Muskmelon DNA was extracted by the CTAB method using plasmid pS-Fom-2 as a positive control and non-transgenic muskmelon as a negative control for the PCR amplification reaction system with the Fom-2 gene amplification program followed by agarose gel electrophoresis detection.

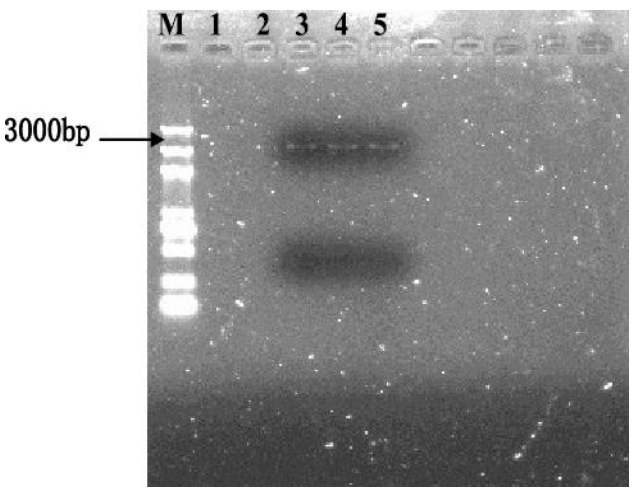
Field resistance identification of resistant plants: When the seedlings grew leaves, randomly selected resistant plants and 50 strains of the control (non-transgenic plants) were treated by root dipping inoculation of *Fusarium oxysporum*, buckle shed insulation and moisture for 7 d.

After days 3, 5, 7, 9 and 11, the transgenic and control leaves were removed for defense enzyme activity determination. Chitinase activity was determined using the method published by Boller (1983); spectrophotometry (Tang Zhangcheng *et al*., 1999) was used to evaluate polyphenol oxidase (PPO) activity; and the callus wood phenol method (Zhu Guanglian *et al*., 1991) was deployed to determine peroxidase (POD) activity.

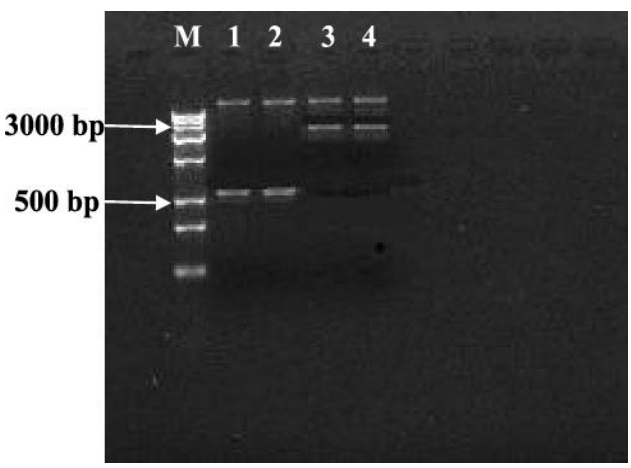
Results

Construction of an antisense plant expression vector of *Fom-2* gene and the acquisition of transgenic melon: The sense and antisense expression vectors were transformed using blue and white plates, and they were randomly selected and screened. A single colony was inoculated into 5 mL containing kanamycin (Kan) LB liquid medium, followed by overnight incubation at 37°C on a shaking platform operated at 170 rpm. Bacterial PCR was used for detection, and the expected bands were obtained (Figs. 3 & 4). The extraction of the plasmid was conducted using double enzyme digestion with BamH I and Xba I. The recombinant expression vector of pS-Fom-2 yielded a 3222 bp band, and the antisense pA-Fom-2 recombinant expression vector yielded a 580 bp band, which was consistent with the expected size. Figure 5 shows that the Fom-2 gene was successfully inserted into the plant expression vector. Then, through the pollen tube pathway in muskmelon incubated for 24 h after pollination, the prepared pS-Fom-2 and pA-Fom-2 plasmid DNA was transferred into muskmelon strains M4-45 and MR-1, resulting in 1585 and 1972 seeds, respectively.

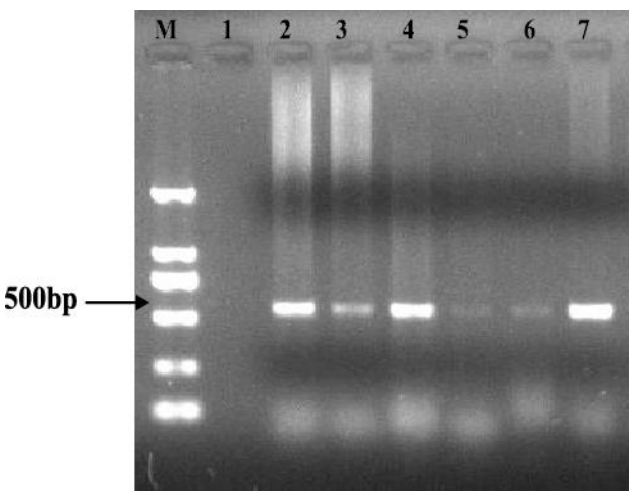
The pollen tube pathway refers to treating plants in the flowering and fertilization stages, during the formation of the pollen tube, via a DNA solution injected into the ovary containing the target gene. The receptor cell genome contains exogenous DNA, and with the development of the fertilized egg, it becomes an individual carrying the target gene. Chinese scholar Zhou Guangyu first proposed the pollen tube pathway theory in the 1970s, and this method reduces the tedious operation of artificial tissue culture, does not require expensive equipment, and can be easily adopted (Wang Yongfeng, 2004).



M: marker5000; 1-2: control; 3-5: Fom-2 gene full-length PCR products
Fig. 3. Expression vector pS-Fom-2 colony PCR detection



M: marker5000; 1-2: pA-Fom-2 double digestion product; 3-4: pS-Fom-2 double enzyme cut product
Fig. 5. Double restriction digestion detection of positive and negative expression vectors.



M: marker2000; 1: control; 2-7: Fom-2 gene fragment PCR product
Fig. 4. Antisense expression vector pA-Fom-2 colony PCR detection

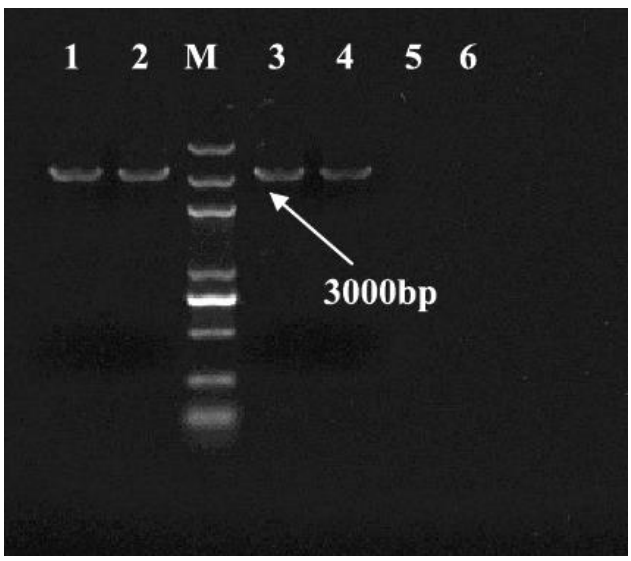
PCR identification of transgenic resistant plants: The pollen tube pathway of muskmelon M4-45 and MR-1 T0 was used to generate muskmelon seedling boxes from seeds using kanamycin (Kan) screening of the resistant plants. The typical appearance of the cotyledon was continuous after 4 d of treatment with kanamycin (Kan) at 1 a.m. every day. Concentrations of 180 mg.L-1 for M4-45 and 170 mg.L-1 for MR-1 were applied evenly to the cotyledons. After 7-10 days of observation of the cotyledons, no yellow and wilted leaves grew from Kan-resistant seedlings, with 52 and 84 strains of M4-45 and MR-1 resistant muskmelon plants, respectively. The results are shown in Table 2.

Table 2. Results of early screening using Kan

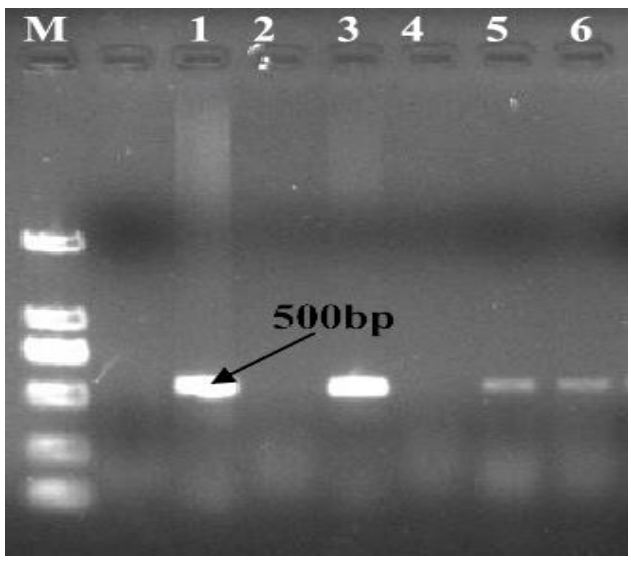
	CK	M4-45	MR-1
Number of seeds	50	1585	1972
Number of green shoots (plants)	0	52	84
Green plantlet rate（%）	0	3.28	4.26

A CTAB extraction method was used to determine the resistance of muskmelon leaves. PCR amplification was conducted using negative control DNA, with the corresponding plasmid DNA used as the positive control, followed by the conversion of muskmelon M4-45 on the *Fom-2* gene and agarose gel electrophoresis (Fig. 6). The results showed that resistant plants could amplify 3222 bp bands consistent with the size of plasmid DNA. However, no bands were amplified from the negative control and water control, indicating that the pS-*Fom-2* gene was integrated into the melon genome.

Using plasmid pA-*Fom-2* as a positive control and non-transgenic muskmelon as a negative control, the 580 bp plasmid DNA was amplified with uniform size, and no bands from the negative control and water control were amplified, indicating that the pA-*Fom-2* gene was integrated into MR-1 muskmelon (Fig. 7).



M: marker5000; 1-2: positive control (plasmid); 3-4: transgenic melon; 5: non-GM melon; 6: water control
Fig. 6. PCR identification of resistant plants.



M: marker2000; 1, 3: positive control (plasmid); 5-6: transformed Antisense Vector MR-1 melon; 2: non-transgenic melon; 4: water control
Fig. 7. PCR identification of MR-1 plant.

3.3 Identification of field resistance and detection of defense enzyme activity in transgenic plants

For further detection of the resistance of transgenic plants to muskmelon wilt, roots were inoculated with a concentration of 1×10^6 spores mL^{-1} from a muskmelon Fusarium spore suspension; roots were at the same age as those of the non-transgenic plants. After day 7, the transgenic plants had a slightly lower incidence than that of the control plants. The incidence rate was 84% in the transgenic plants and 96% in the control plants.

To identify the resistance mechanism of the transgenic plants, the defense enzyme activity was determined, and the results are shown in Figures 8–10. In the treatment group and control group, the levels of plant chitinase, peroxidase and polyphenol oxidase increased, with the increase continuing until the time of death. We observed a trend of significantly higher enzyme content in the transgenic plants in vivo than that of the control plants. In the early stage of infection (3 and 5 d), the transgenic plants and control plants exhibited small changes in polyphenol oxidase activity. At 7 d after treatment, the content of polyphenol oxidase in the transgenic plants was significantly higher than that of control plants.

Discussion

Genetic transformation using the pollen tube pathway: The pollen tube pathway is a transgenic breeding technology at the molecular level. Compared with the traditional breeding process of natural selection, the technology has significant advantages based on the introduction of exogenous gene orientation. Resistance genes to plants that lack wild-type genes can be developed, and combined with traditional breeding, transgenic crops can be bred, resulting in new varieties and new materials. Since its development, the pollen tube pathway has been used in Chinese corn (Wang, 2002), cotton (MA, 2004), wheat (Yin, 2004), soybean (Liu, 2006), rice (Meng, 2006), muskmelon (Haas, 2007), tobacco (Li *et al.*, 2008), cucumber (Zhang, 2009), tomato (Chen, 2010) and peanut (Liu, 2010), in addition to 60 other types of crops and vegetables.

In this study, the full-length sequence of *Fom-2* was constructed using the pollen tube pathway, and transgenic lines with increased resistance were obtained by constructing the corresponding plant expression vector. Because genetic transformation was conducted by the pollen tube pathway, the percentage of transgenic melons and the number of seeds derived from a single melon decreased during ovary injection. A possible reason was that the ovary injection wounds increased susceptibility to pests and mildew at the late stage, which affected the fruiting rate and seed number. Therefore, when ovary injection is conducted, the contamination and damage to the ovary are minimized.

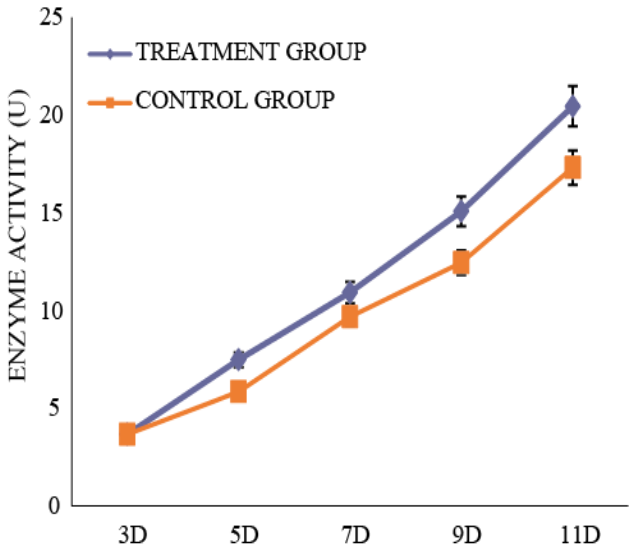


Fig. 8. Chitinase activity curve.

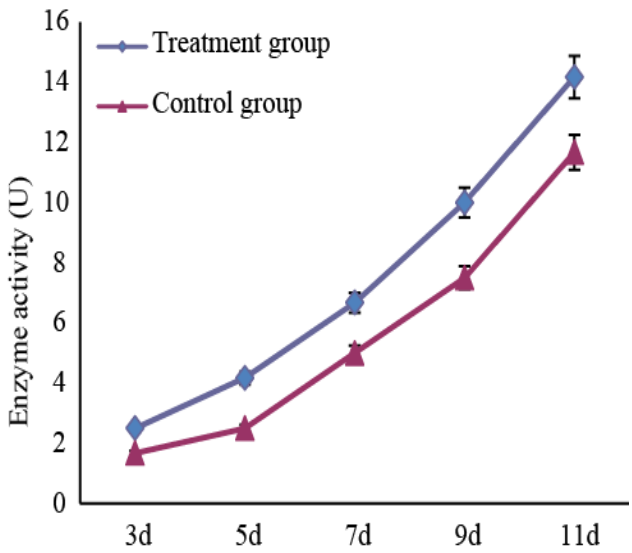


Fig. 9. Peroxidase activity curve.

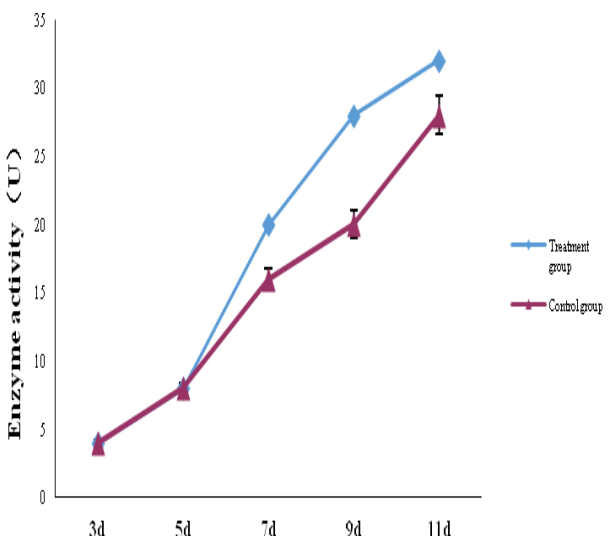


Fig. 10. Polyphenol oxidase activity curve.

Genetic transformation of other genes in *Cucumis melo*: Melon cultivation in China has a long history, and melons are planted in several regions. Good varieties have been developed through conventional breeding methods for different planting areas with different features, but the species reproductive barriers seriously affect the application of conventional breeding methods. Compared with traditional breeding, superior resistance is obtained through the genetic transformation method, and many genes have been transferred into muskmelon varieties. Wang (2011) was cloned from *M01-3* to *Psy* in the Muskmelon Fruit gene by RT-PCR technology. The product was digested using the expression vector PBI121 and double enzyme digestion. The recombinant plasmid pMD18-T-*Psy* was reverse ligated using an antisense expression vector, and the *Agrobacterium*-mediated genetic transformation of muskmelon was achieved. Several methods have been used to generate Kan-resistant transgenic plants. Bard (2012) used RT-PCR to clone genes from Hetao muskmelon varieties of mature fruit using the alpha -Man gene cDNA fragment. The binary expression vector of the alpha -Man gene plants was constructed by the pollen tube pathway method for genetic transformation of muskmelon, and the results indicated that the transgenic alpha -man RNAi vector of Muskmelon Fruit Storability and the shelf life were significantly extended. Cheng (2013) used RT-PCR to clone a cDNA sequence from a muskmelon powdery mildew susceptible gene *CmMLO2*, to construct an RNAi expression vector pFGC1008-*CmMLO2* after transformation using muskmelon leaf discs. After obtaining the PCR-positive plants, the results showed the identification of powdery mildew inoculation and the development of transformed plants that were resistant to powdery mildew, as evidenced by ihpRNAi knockdown of *CmMLO2*, which resulted in the formation of powdery mildew resistant materials of muskmelon.

Identification of transgenic plants: Transgenic plants must be genetically identified to test whether the target gene is transferred into the recipient material. PCR testing showed that the resistance gene Fom-2 was successfully transferred into the melon, and the identification of resistance to *Fusarium oxysporum* with special inoculation showed that the resistance of the transgenic melon to Fusarium wilt increased. The positive kanamycin-based test and PCR-positive detection of transgenic muskmelon are problems because these results were not consistent with the analysis results. The PCR results from kanamycin resistance analysis were not consistent with the results from the study conducted by Zhu (2000). Apparently, the inconsistent results could be due to the presence of DNA during the extraction of phenolic compounds from plant cells, which forms irreversible complexes, thereby affecting the test results. Alternatively, injection of exogenous DNA into the ovary might cause degradation of plant nucleic acids and release enzymes, which could aid the formation of incomplete gene fragments, thereby affecting the normal expression of the gene (Haas Aguilar, 2007).

Fom-2 is an important gene in muskmelon. Wang (2014) studied the specific spatiotemporal expression characteristics of muskmelon Fusarium infection using fluorescence quantitative PCR technology-based research of the Fom-2 gene. This gene is induced by Fusarium, and after 24 h, the expression begins to be up regulated in resistant varieties, with expression reaching a peak shortly thereafter. The gene plays a role in signal transduction, the defense system, and the resistance to pathogenic bacteria in the disease process. Using comparative genomics of muskmelon *Fusarium oxysporum*, Schmidt (2015) analyzed biotype strains to identify a candidate gene and the AVRFOM2 gene by a whole genome comparison approach. The gene encoding avirulence proteins is recognized by Fom-2, resulting in resistance. It is important to understand the recognition and conduction behavior of the corresponding role of resistance genes and the disease resistance protein signal. Understanding the role of avirulence genes in plant disease resistance is also important in assessing the effectiveness and durability of disease resistance. Additionally, it is of great importance to determine the effective use of resistance genes that confer a high degree of disease resistance and durability to provide the foundation for the genetic improvement of crops. The process of plant resistance to viral infection is related to several genes, and the determination of the expression of the key genes related to improving the regulatory mechanism of muskmelon resistance through biological engineering requires further research.

Acknowledgments

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