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EXPRESSION ANALYSIS OF *FUSARIUM* WILT RESISTANCE GENE IN MELON BY REAL-TIME QUANTITATIVE PCR

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Abstract

Melon *Actin* gene was used as a reference gene, to explore the gene expression profiles of the *Fom-2* gene in roots, stems, and leaves of melon MR-1 under induction by *Fusarium oxysporum* f. sp. *melonis*. Monitoring using real-time quantitative PCR showed similar accumulation patterns of *Fom-2* in roots, stems, and leaves over the observation period of 1 to 11 days; the expression level in stems was the highest. The expression of the *Fom-2* gene was strengthened by the prolongation of induction time. In stems, the expression of *Fom-2* was 5.737 times higher than in the control at three days; in roots, expression of *Fom-2* was 5.617 times higher than in the control at five days. Similarly, the expression of *Fom-2* in leaves obviously increased. It was 4.441 times higher than in the control at 5 days. The expression of *Fom-2* was non-tissue specific, up-regulated under induction by *Fusarium*, and related to early resistance to *Fusarium* wilt.

Introduction

Fusarium wilt is an important disease of melons around the world, caused by *Fusarium oxysporum* Schlecht. f. sp. *melonis* (Leach et Currence) Snyder et Hansen (Sun *et al*., 2000; Zuniga *et al*., 1997, Namiki *et al*., 1998, Kurt *et al*., 2002). Over recent years, with the market demand for melon, the area under cultivation has expanded, and cultivation methods have diversified. The disease risk from *Fusarium* is thus increasing year by year. Yearly losses to *Fusarium* have been calculated as 10-30%, as high as 100% (Sherf & Macnab, 1986).

The use of improved melon cultivars with excellent resistance genes is an effective and economical method of controlling *Fusarium* wilt. Currently, the melon biotypes identified in the international wilt include four physiological races (0, 1, 2, and 1.2) (Han *et al*., 2009). Melon resistance genes have been found and reported. They are the *Fom-1* gene (resistant to races 0 and 2 but susceptible to race 1), the *Fom-2* gene (resistant to races 0 and 1 but susceptible to race 2) (Risser, 1973), and the *Fom-3* gene (resistant to races 0 and 2 but susceptible to race 1) (Zink & Gubler, 1985).

Many researchers are currently focused on the markers, isolation, and cloning of *Fusarium* wilt resistance genes in melon; acquiring molecular markers for *Fom-1* and *Fom-2*, and isolating and cloning *Fom-2* (Zheng *et al*., 1999; Zheng & Wolff, 2000; Luo *et al*., 2001; Touyama & Kanbe, 2002; Joobeur *et al*., 2004; Nakazumi & Hirai, 2004; Brotman *et al*., 2005; Perchepied *et al*., 2005; Oumouloud *et al*., 2008; Tezuka *et al*., 2009). There are 5 sequences of *Fom-2* (Joobeur *et al*., 2004) in GenBank, cloned from different varieties of melon. There are some differences between the sequences, but the homology is high. Although the genes above have been cloned, there remain many unknowns. How is the *Fom-2* gene involved in melon resistance to *Fusarium* wilt? What are the expression features and functions? This knowledge would provide a theoretical basis for the implementation of genetic engineering and molecular breeding of melon resistance to *Fusarium*.

We used melon MR-1 which is highly resistant to *Fusarium* wilt. We acquired the cDNA sequence of the resistance gene *Fom-2* by homologous cloning, selected the melon *Actin* gene as the reference gene, and analyzed the timing and expression characteristics of the *Fom-2* gene in the process of *F. oxysporum* f. sp. *melonis* infection. Using real-time quantitative PCR, we clarified the function of the *Fom-2* gene in melon resistance to *Fusarium* wilt. We have provided a reference to enable further definition of the breeding value of the gene in melon resistance to *Fusarium* wilt, and the use of future genetic modification of melons.

Materials and Methods

Melon materials and melon *Fusarium* wilt: Melon MR-1 was provided by Prof. Yong Xu at the Chinese Academy of Agricultural Sciences; obtained from self-cross-breeding in the Melon and Watermelon Molecular Breeding Laboratory of Northeast Agricultural University. The *Fusarium* strain (identified as race 1) was purified and identified at the Melon and Watermelon Molecular Breeding Laboratory of Northeast Agricultural University.

Reagents: TRIZOL was purchased from Invitrogen; Revertaid cDNA Synthesis Kit was purchased from Fermentas; the Master Mix was purchased from Aidlab Biotechnologies; DNA polymerase, dNTPs, and a DNA molecular weight marker were purchased from TaKaRa Biotechnology. The chloroform and isoamyl alcohol were both of analytical grade.

Primers for the *Fom-2* gene and the reference gene: We chose the *Fom-2* gene (GenBank accession number AY58385), cloned from melon, as a target gene to analyze, and the *Actin* gene (Guan, 2007) as a reference gene. Primers were designed using Primer Premier (version 5.0) (Premier company, Canada), and synthesized by the Shanghai Biological Engineering Company (Table 1).

Table 1. Primers for the *Fom-2* gene and the reference gene.

Gene name	Sequences (5'–3')
Fom-2	TTGGCTTCCTCTTTACTT
	GTCTATTGTTTCGCTTCA
Actin	TCTATTCCAGCCATCTCTC
	GACCCTCCAATCCAAAC

Melon cultivation: We sowed 80 melon MR-1 seeds in the garden of Northeast Agricultural University, after seed sterilization and soil fumigation.

***Fusarium* pathogen inoculation:** When seedlings were at the two leaves stage, we inoculated with *F. oxysporum* using the dipping method (Wang *et al*., 2000), and placed a temporary shed around them to maintain consistent temperature and humidity.

RNA extraction and reverse transcription: Total RNA of roots, stems, and leaves inoculated by *Fusarium* at different times was extracted using TRIZOL reagent, Samples were ground in liquid nitrogen, and then mixed with TRIZOL, centrifuged at 4°C, and centrifuged at 12,000 ×g for 5 min. The supernatant was retained and the pellet discarded. The sample was kept at room temperature for 15 min, then chloroform was added at a ratio of 5:1. The lid was placed on the sample, and it was then shocked for 15 s, incubated on ice for 15 min, and then centrifuged at 12,000 ×g for 15 min. The supernatant was retained, and transferred to a clean centrifuge tube. An equal volume of ice-bathed isopropanol was added, mixed by inversion, and samples were kept at -20°C for more than 20 min, then centrifuged at 12,000 ×g for 10 min. The RNA formed a flaky precipitate at the bottom of the tubes.

The supernatant was removed, the RNA pellet was washed once with 75% ice-bathed ethanol, and the centrifuge tubes were inverted and washed. Samples were shocked, sediment was suspended if possible, and then centrifuged at 12,000 ×g for 5 min. The supernatant was again removed, and the samples were dried on a clean bench. The RNA was dissolved in 30 µL of DEPC, and stored at -70°C.

Reverse-transcribed using Revertaid cDNA Synthesis Kit k1621. We tested the quality of cDNA compared with the reference gene after transcription.

Real-time quantitative PCR: The 25 µL PCR amplification mixture contained 12.5 µL of 2×SYBR qPCR Mix, 1 µL of cDNA as template, 0.5 µL of each 10 µmol.L^{-1} PF and PR primers, and DEPC-treated water. The PCR protocol was as follows: 94°C for 3 min, 94°C for 1 min, 55°C for 30s, 72°C for 1 min, 80°C reading plate for 1s, for 30 cycles, and a final extension of 72°C for 7 min. Each sample was repeated four times, and a melting curve was obtained using IQ5 (Bio-Rad, USA) after the PCR reaction (Wang *et al*., 2012; Xie *et al*., 2013).

Data analysis: The reference gene *Actin* was used as the standard for relative quantification, the comparative 2-□□Ct method was used to analyze the relative transcription levels of the Fom-2 gene: relative expression = 2-□□Ct = 2-[(E-F)-(A-B)] = 2(F-B)-(E-A), where A is the Ct value of tested genes before intervention; B is the Ct value of the reference gene before intervention; E is the Ct value of the tested genes after intervention; and F is the Ct value of the reference gene after intervention. The Ct value is that four times of repeated difference does not exceed the average of the 0.5 Ct value.

Results and Discussion

Analysis of the melting cure of the *Fom-2* gene in melon MR-1: Figure 1 shows that both the target gene *Fom-2* and the reference gene *Actin* were amplified, and reached their peak at 81°C and 83.5°C, without other products or any primer-dimers. The entire experimental process was without contamination, and the target and reference genes were stably expressed.

Analysis of timing and spacing expression of *Fom-2* gene in melon: The *Fom-2* gene was expressed in roots, stems, and leaves, but there were significant differences in relative expression levels between the different tissues. The relative expression from highest to lowest was stems>roots>leaves.

The relative expression levels of the *Fom-2* gene in the different organs showed essentially the same increasing trends after inoculation with *Fusarium* wilt (Figs. 2, 3, and 4). The relative expression levels of the *Fom-2* gene in melon roots and leaves increased sharply from the fifth day, and remained rising. The relative expression levels of the *Fom-2* gene in melon stems increased sharply from the third day, and relative expression levels were higher than those in the roots or leaves.

Duncan 's new multiple range test was used to analyze *Fom-2* gene expression levels (Table 2), under induction by the *Fusarium* pathogen. As time progressed, the relative expression levels of *Fom-2* in roots and stems increased significantly in each treatment. However, there was no significant increase in leaves on the first, third, ninth, or eleventh day.

Resistant genes to *Fusarium* wilt in melon: There are three known melon genes resistant to *Fusarium* wilt. These are *Fom-1* (resistant to races 0 and 2, but susceptible to race 1.2), *Fom-2* (resistant to races 0 and 1 but susceptible to race 2 and 1.2) (Risser 1973), and *Fom-3* (resistant to races 0, 1, and 2 but susceptible to race 1.2); all are dominant genes (Zink & Gubler, 1985). Using the gene library and molecular markers identified by Wang *et al*., (2000) as tightly linked with the *Fom-2* gene, Joobeur *et al*., (2004) isolated and cloned the *Fom-2* gene; this was the only access to the cloning resistance gene in melon. In 2005, Zhao *et al*., following the homologous features of the *Fom-2* gene, isolated a cDNA fragment related to melon resistance genes, using RT-PCR technology. This was named X-*Fom-2,* identified by molecular hybridization analysis, and found to exist in multiple copies in susceptible and resistant varieties. Northern blotting showed that the *Fom-2* gene does not express in susceptible varieties, but expresses greatly in resistant varieties, and that expression is induced by pathogens.

We firstly studied the timing and spacing of expression characters of the *Fom-2* gene during the process of induction by *Fusarium* wilt, using real-time quantitative PCR. We made preliminary quantitative observations of expression of *Fom-2* in induction with *Fusarium oxysporum*, and uncovered functions of the gene in melon resistance to *Fusarium* wilt, and in order to provide reference for further study.

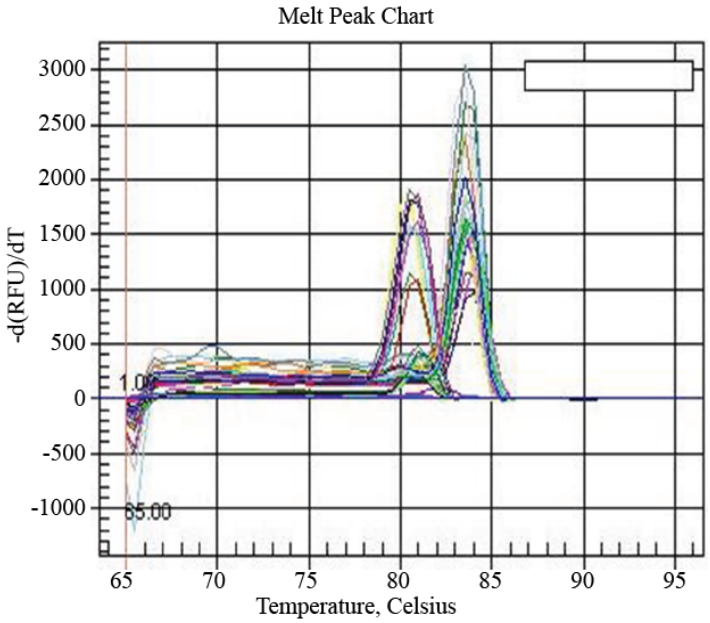

Fig. 1. Melting curve of *Fom-2* and *Actin*.

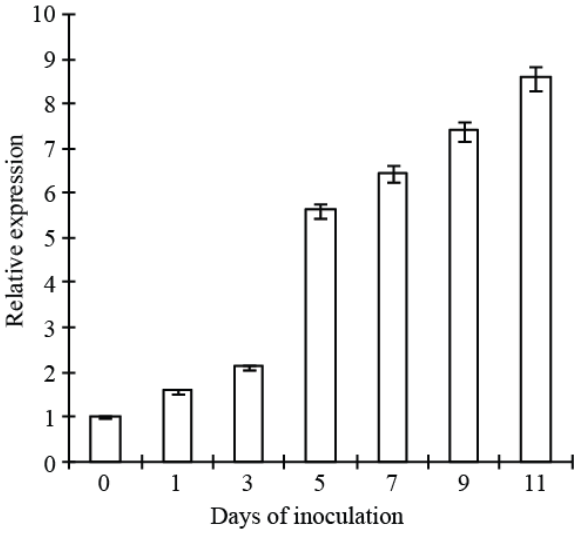

Fig. 2. Relative expression levels of the *Fom-2* gene in roots of melon MR-1 inoculated with wilt at different times.

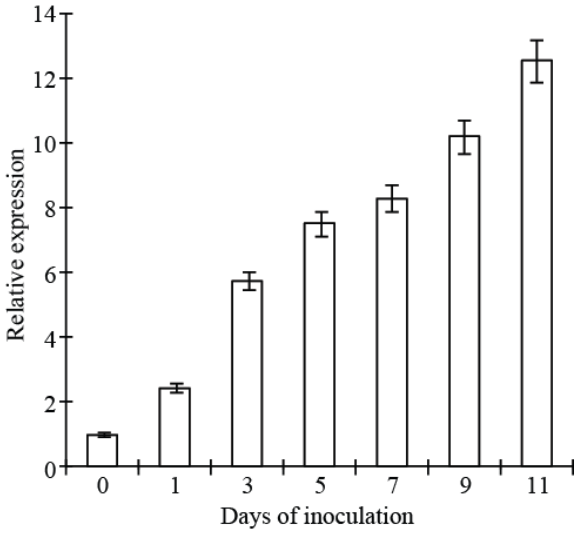

Fig. 3. Relative expression levels of *Fom-2* gene in stems of melon MR-1 inoculated with wilt at different times.

Table 2. Time and space expression characteristics analysis of the *Fom-2* gene in melon.

Days of inoculation	Roots	stems	Leaves
0	1.002 ± 0.002 aA	1.002 ± 0.002 aA	1 ± 0.002 aA
1	1.5827 ± 0.0242 bB	2.4397 ± 0.1841 bB	1.1913 ± 0.0637 bB
3	2.117 ± 0.1107 cC	5.7487 ± 0.1695 cC	2.263 ± 0.0471 bB
5	5.6287 ± 0.1087 dD	7.5257 ± 0.1337 dD	4.4413 ± 0.1537 cC
7	6.4253 ± 0.1766 eE	8.302 ± 0.0875 eE	5.5253 ± 0.2762 dD
9	7.4033 ± 0.1753 fF	10.204 ± 0.0708 fF	5.707 ± 0.2307 eE
11	8.5777 ± 0.0665 gG	12.5300 ± 0.0700 gG	7.0457 ± 0.0665 eE

Note: Different small letters represent significant differences at the 5% level. Different upper-case letters represent significant differences at the 1% level

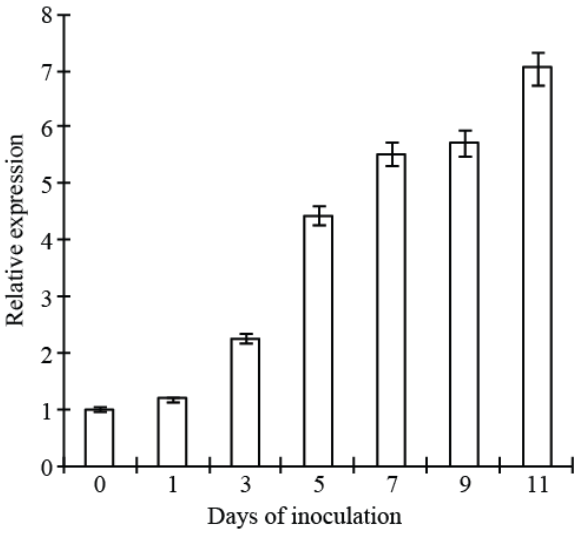

Fig. 4. Relative expression levels of *Fom-2* gene in leaves of melon MR-1 inoculated with wilt at different times.

The SYBR Green I real-time quantitative PCR of *Fom-2* gene in melon: This experiment successfully established the usefulness of the SYBR Green I real-time quantitative PCR of *Fom-2* gene in melon, compared with Northern blotting and semi-quantitative PCR (Zhang *et al*., 2007). Real-time quantitative PCR provides a wide range of quantitative PCR detection, amplification efficiency, specificity, and short detection cycle. The method uses a closed tube operation, eliminating the problems of amplified product contamination and false positives in conventional PCR methods. Using real-time quantitative PCR can therefore provide quick and accurate detection of *Fom-2* gene expression in melon. This has provided a foundation for the future study of melon gene function and molecular breeding.

The character of *Fom-2* in melon and further research questions: Plants have gradually formed a set of disease-resistance mechanisms over their long-term co-evolution with pathogens. Plants produce antibacterial compounds after pathogen infection. However, due to different emergency response speeds, synthesis of disease-resistant compounds differs greatly, meaning that some plants are resistant, while others are susceptible (Wang *et al*, 2011; Chen *et al*., 2012).

Fom-2 is a NBS-LRR-type R gene. These genes contain two conserved sections of coding NBS and LRR protein. NBS does not exist independently, but forms NBS-LRR with LRR. LRR is closely related to protein interactions and signal transductions. In plants, proteins containing LRR play important roles in cell growth and disease resistance responses (Kobe & Deisenhofer, 1994).

The *Fom-2* gene is of importance as it begins up-regulation in resistant varieties 24 h after inoculation of *Fusarium oxysporum*, and quickly reaches peak expression, to achieve signal transduction, start the defense system, and resist the pathogen. The gene does not express in susceptible plants induced by the pathogen (Zhao, 2005).

Many further research questions remain. These include the sub-cellular localization of the *Fom-2* gene in the melon, how to trigger the melon pathogen defense system, and how to regulate gene expression.

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