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Screening some Iranian Muskmelon Landraces for Resistance Against Fusarium Wilt Disease using Molecular Markers

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Abstract
Fusarium wilt is one of the most destructive diseases of muskmelon (*Cucumis melo* L.), which is an economically important disease worldwide causes yield losses in muskmelon growing areas. One of the most effective controlling measures to prevent Fusarium wilt is through host resistance by using resistance genes. We used developed molecular markers for *Fom-2* gene, which confers resistance to race 1 of Fusarium in muskmelon, to screen muskmelon landraces in Khorasane-e-Razavi, Iran. After validation of the markers on a differential set of resistant and susceptible lines, we identified STS312 marker as the polymorphic and easy-to-score marker. Then we used STS312 to genotype plants from five different landraces. Our results suggest that resistance allele of *Fom-2* gene is present in two landraces: Eyvankey and Mashhadi. These landraces can be used by muskmelon breeders to enhance resistance to Fusarium wilt in muskmelon.

Keyword: *Cucumis melo*, Fusarium wilt, landrace, molecular markers.

Introduction

Muskmelon (*Cucumis melo* L.) is a diploid plant species (2n = 2x = 24) in *Cucurbitaceae* family, and is important for its specific biological properties and for its economic value (Joobeur et al., 2004). The cultivation area and production of melon is estimated to be around 85000 ha and 1.7 million tones, respectively in 2018 (Nations., 2018). Since Iran is suggested as one of the center of origin of muskmelon (Raghami et al., 2014), different landraces of muskmelon is traditionally cultivated in different regions of Iran. These landraces are excellent resource for muskmelon breeding projects.

A major limiting factor in melon production is diseases caused by fungal pathogens. The Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *melonis* reduce yield and fruit quality by infecting muskmelon roots and blocking the uptake of water and nutrients, eventually killing the root and above ground tissues (Oumouloud et al., 2013). There are five different races of *F. oxysporum* f. sp. *melonis* (Fom) named as FOM0, FOM1, FOM2, and FOM1.2. Because the pathogen can survive in the soil for decades, it is a difficult to control

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disease through fungicide applications. Therefore, breeding resistant cultivars is probably the most effective and practical strategy for controlling this pathogen (Joobeur et al., 2004). Until now five resistance genes for Fusraium have been identified in muskmelon germplasm. The *Fom-1*, originally identified in the cultivar Doublon, confers high levels of resistance to FOM0 and FOM2, whereas the *Fom-2* gene, originally identified in the cultivar CM17187, confers high levels of resistance to FOM0 and FOM1 (Risser et al., 1976). *Fom-1* and *Fom-2* both encode NBS-LRR proteins (Joobeur et al., 2004; Brotman et al., 2013). A recessive gene, *fom-4*, is also reported to be conferring resistance to races 0 and 2, along with *Fom-1* gene (Oumouloud et al., 2013). *Fom-2*, which is located on chromosome 11, is a 3-kb gene (GenBank Accession number: DQ287965.1) encoding a 1073 amino acid peptide. Resistance to race 1.2 is complex and appears to be under polygenic control (Perchepied et al., 2005).

DNA molecular markers facilitate breeding projects significantly. Selection based on markers has several advantages over conventional phenotypic selection including higher reproducibility and independence of environmental effects, independence of growth and developmental stage of the plants, and being able to discern heterozygous and homozygous plants. Markers are particularly very helpful for disease resistance breeding projects where many different plant genotypes have to be challenged with different races of pathogens to screen for resistance against the pathogen. Plant response to pathogens is very often developmental stage-dependent and very much influenced by the environment. Molecular markers tightly linked to Fusarium wilt resistance genes are highly valued in muskmelon breeding. Several markers have been reported for the *Fom-2* gene. AM and FM were the first markers developed for *Fom-2* by converting RAPD and AFLP markers linked with *Fom-2* (Wang et al., 2000). These flanking markers are 240 kb and 167 kb apart from the gene. STS178 and STS312 are 241 Kb and 90 Kb apart from the gene, respectively (Joobeur et al., 2004). FM, CAPS2, Fom2-R409, and Fom2-S253 are gene-based markers (Wang et al., 2011; Sousaraei et al., 2018).

The FOM1 is prevalent in Khorasane-e-Razavi province in Iran, but the other races are not reported in this region yet (Banihashemi, 2010). In the present study, we tested different molecular markers that have been introduced for *Fom-2* resistance gene, and then use them for screening populations of landraces of muskmelon in Khorasane-e-Razavi, Iran.

Material and Methods

Plant Materials

Differential set for the Fusarium wilt disease including Doublon, CM17187, Isabelle and Charentais T lines, along with Samsuri cultivar were received from Falat seed company, Iran. Doublon is resistant to FOM0 and FOM2 but is susceptible to FOM1 and FOM1.2, and CM17187 is resistant to FOM0 and FOM1 but is susceptible to FOM2 and FOM1.2. Charentais T and Isabelle are susceptible and resistant lines, respectively, to all the four races of Fusarium. Samsuri is resistant to FOM1. Seeds from five landraces of muskmelon in Khorasane-e-Razavi including Mashhadi, Eyvankey, Nasrabadi, Mahvelati Type 1, and Mahvelati Type 2, were harvested from single fruits in summer 2018. These landraces are all belonged to inodorous group. The fruits of representative individual of the landraces are shown in Figure 1.

DNA markers

Three markers have been introduced for *Fom-2* gene, two STS markers (Joobeur et al., 2004) and one gene-based CAPS marker (Wang et al., 2011). Primers for these markers were synthesized by Macrogen company, South Korea (Table 1).

Fig. 1. Fruit morphology of different muskmelon landraces. Representative fruits of Eyvankey (E), Mashhadi (M), Nasrabadi (N), Mahvelati type1 (MT1), and Mahvelati type 2 (MT2) are shown.

Table 1. Description of DNA markers that are used in this study.

Marker Name	Primer sequences (5'-3')	Reference
STS178	F:TTCGTTCATTACTGCCGTAGG R: TCTGTGTTCCCTACCCCAAC	(Joobeur et al., 2004)
STS312	F: GGAGGATTTGGGAAGTGAG R: TGTCCATACCTCCTCCAAGC	(Joobeur et al., 2004)
CAPS2	F: GGAAGTGAGGTGTTGAATT R: TACACATTGGTCCGTTAGAC	(Wang et al., 2011)

DNA extraction

Genomic DNA was isolated from cotyledons using a modified CTAB method (Fulton et al., 1995). In brief, 100 mg of cotyledons was ground in liquid nitrogen to a fine powder and transferred to 1 mL of the extraction buffer (20 mM EDTA pH 8, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB). The mixture was incubated at 65 ℃ for 1 h with occasional agitation. After 5 min incubation on ice, 250 µL chloroform/isoamylalcohol 24:1 (v/v) was added and mixed well, and then centrifuged for 10 min at 10000 g. The aqueous phase was transferred to a new tube and 0.6 volume of isopropanol was added to each tube and mixed by inverting. Tubes were centrifuged at 10000 g for 10 min, and the pellet was washed in 70% ethanol and dissolved in sterile H_2O containing 10 µg mL^{-1} *RNase* A.

PCR amplification

Concentration of DNA was adjusted to approximately 80 ng μL^{-1}. For PCR, 20 µL reaction mixtures comprised of 10 µL of 2X Taq polymerase premix (Cat#C101081, Parstous Biotechnology, Iran), 0.5 µM of each primer and 80 ng of DNA were prepared. The touchdown PCR profile consisted of an initial denaturation step at 94 °C for 4 min followed by 5 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s, and 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. During the first 5 cycles, the annealing temperature was decrease 1 degree per cycle. The program ended with a final extension step of 72 °C for 5 min. The PCR products were electrophoresed on 2% agarose gels with 1% TAE buffer and visualized under UV transilluminators (UVP, USA).

Results

To find resistance sources to FOM1 in muskmelon landraces in Khorasan-e-Razavi province, we tested the applicability of the molecular markers reported in the literature for *Fom-2* resistance gene. In the next step we used

the polymorphic markers on plants from five populations of muskmelon landraces.

Molecular markers for Fom-2 gene

There were three markers developed for *Fom-2* gene, STS178, STS312 (Joobeur et al., 2004), and CAPS2 (Wang et al., 2011). We used the markers on differential set of muskmelons with different responses to FOM1. CAPS2, which is a marker designed based on the *Fom-2* gene, did not result in a good amplification in our materials. Therefore, we omitted this marker from further experiments. Markers including: STS312 and STS178 on the other hand, gave the expected polymorphic pattern between resistant and susceptible genotypes (Fig. 2). STS312 showed a 367 bp allele in resistant genotypes and a 322 bp allele in susceptible ones. STS178 showed 241 bp allele in resistant genotypes and 367 bp allele in susceptible ones. STS178 and STS312 are about 241 Kb and 90 Kb far from the *Fom-2* gene. STS178 is closer to the *Fom-2* gene, we used this marker for screening the landraces.

Resistant genotypes in landraces

Khorasane-e-Razavi province is one of the main regions for muskmelon cultivation in Iran, and many different landraces are cultivated by farmers there. We collected seeds from five of the mostly used landraces in the province and genotyped them for possible source of resistance to FOM1. The results showed that the resistance allele of *Fom-2* is present in Eyvankey and Mashhadi populations (Fig. 3). The resistance allele was observed in heterozygout state in 4 out of the eight tested plants in Eyvankey. In Mashhadi, all the genotyped individuals were homozygote for the resistance allele. The other three populations showed susceptible allele.

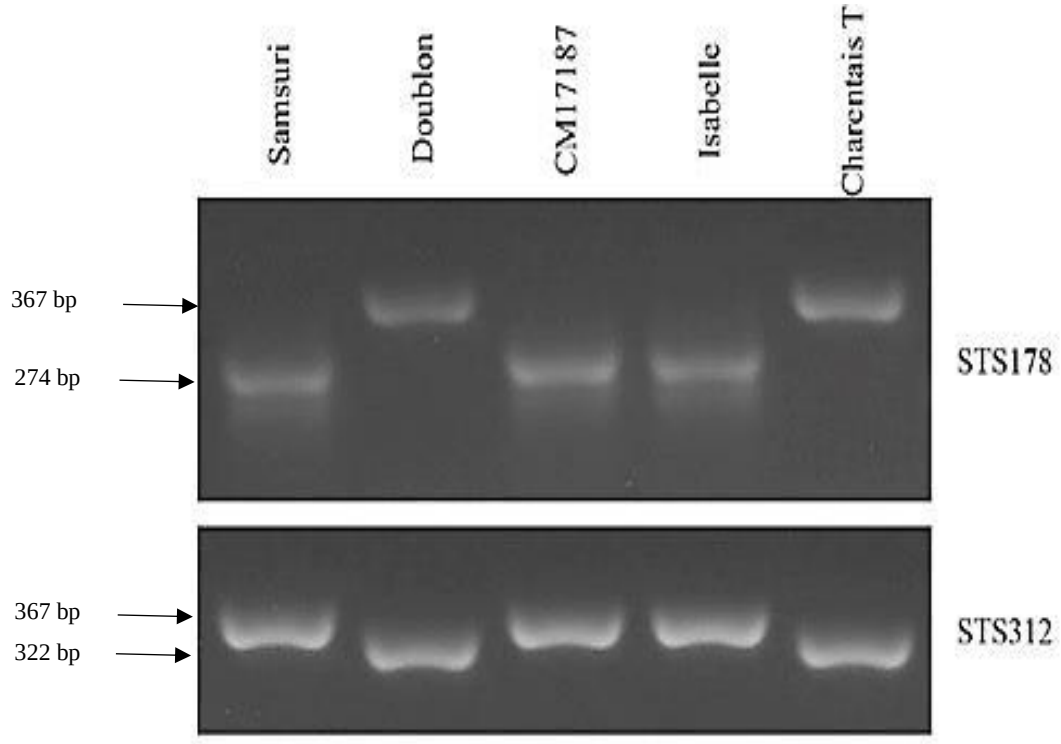

Fig. 2. Polymorphism of STS178 and STS312 markers on the melon differential set. CM17178, and Isabelle are resistant and Doublon and Charentais T are the susceptible lines to FOM1. The Samsuri cultivar is included in the experiment as it was claimed to be the resistant line.

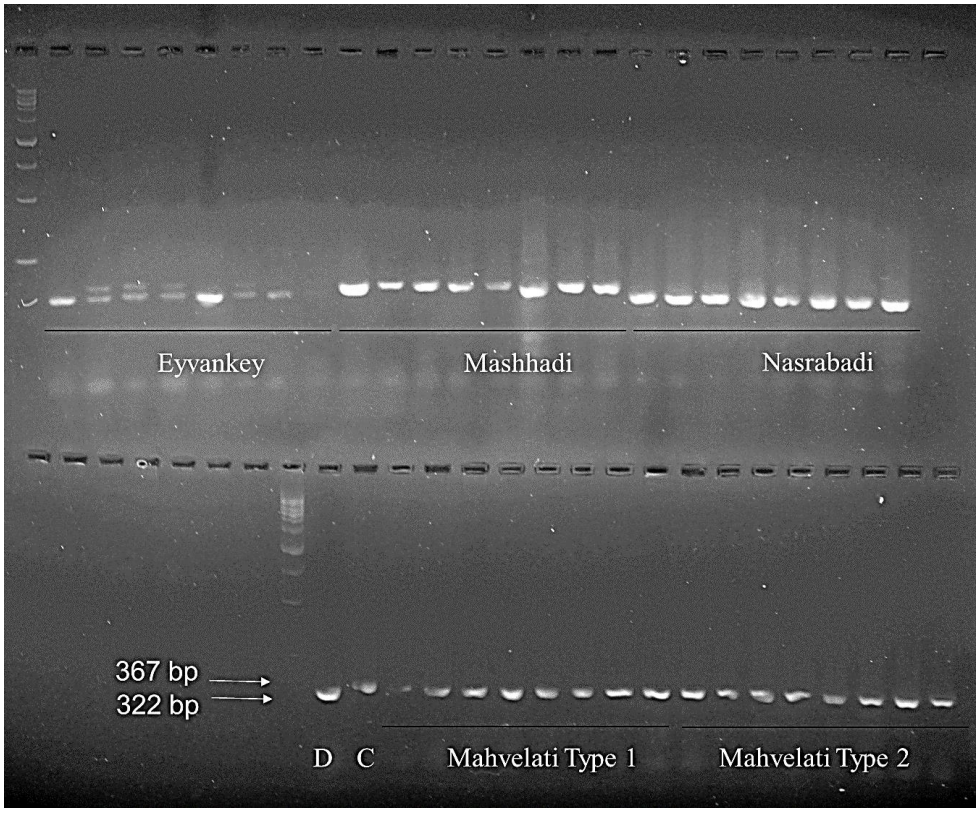

Fig. 3. Screening five landraces of muskmelon using STS312 marker. From each landrace eight plants were used for genotyping by STS312. Resistance allele is present in 4 plants of Eyvankey and all plants of Mashhadi landrace. The resistance (367 bp) and susceptible (322 bp) alleles are shown in CM17187 (C) and Doublon (D).

Discussion

Fusarium wilt is one of the main causes of production loss in muskmelon cultivation worldwide, and therefore, breeding efforts for resistance to this pathogen is highly valuable. For breeding against Fusarium wilt two different strategies can be taken. On straightforward approach is to transfer the resistance genes from the breeding resistant lines, like CM17187 and Isabelle (Risser et al., 1976), into elite breeding lines. After several rounds of backcrossing the characteristics of the recurrent parent is substantially restored (Sousaraei et al., 2018). The second strategy is to explore the native melon landraces to find resistant plants (Banihashemi 2010; Chikh-Rouhou et al., 2010; Madadkhah et al., 2012). Screening of the landraces is advantageous because resistant genotypes can be developed to new cultivars with good adaptability to the region. Also it may result in discovery of new resistance alleles. Thus, in the current work we explored presence of *Fom-2* locus in some of the landraces in Khorasane-Razavi.

Artificial inoculation of the FOM spores on melon genotypes is the common procedure for screening for Fusarium resistance (Banihashemi, 2010; Chikh-Rouhou et al., 2010; Madadkhah et al., 2012). This direct phenotyping method is tedious and usually affected by the developmental stage of the plant, inoculation procedure, and environmental factors. It is reported that the plant responses are different in different methods and concentration of inoculation (Banihashemi and Dezeeuw, 1973). Therefore reproducibility of such screening procedures is not as high as selection based on molecular markers. Furthermore, in such experiments the heteroenousity of the melon landraces is not carefully

considered. In such assessments 4 to 12 are taken from each landrace and a mean of disease index reported as the resistance level (Banihashemi, 2010; Chikh-Rouhou et al., 2010; Madadkhah et al., 2012). Muskmelon produced two forms of flowers, staminate and hermaphrodite, and only the pollens from staminate flowers are involved in pollination (Belavadi, 2019). This make the muskmelon a cross pollination plant species, and thus, muskmelon landraces are expected to be highly heterogenous. The landraces are diverse populations with different phenotypic characteristics. Accordingly, genetic diversity analysis in Iranian melon landraces revealed that 69-87% of the genetic variation is due to within population variations (Raghami et al., 2014; Maleki et al., 2018). It is also worth mentioning that often local farmers cultivate several landraces in the same field, which increases the genetic diversity in the landraces. While we detected resistance alleles to FOM1 in Eyvankey and Mashhadi landraces, disease tests had scored them as intermediate resistant (Madadkhah et al., 2012) or susceptible (Banihashemi, 2010). The above-mentioned heterogenous nature of the landraces is probably the reason for these contrasting results. Indeed, in our experiments, even plants derived from different fruits collected from the same field showed different marker genotype.

We used three markers for *Fom-2* gene on the differential set. STS178 and STS312 were polymorphic on the differential set, as expected, and because STS312 was the closer marker to *Fom-2* we used this marker for screening the landraces. This marker gave a discernable banding pattern between resistant and susceptible alleles. Gene-based markers are preferred for marker-assisted selection as there is no chance of recombination between the marker and the target gene. Such gene-based markers have been developed for *Fom-2* gene based on the leucine-rich repeat domain of the gene (Wang et al., 2011; Sousaraei et al., 2018). We used the CAPS2 marker in this study, but it amplified properly in our experiments, and therefore, it was not used for the screening part. Given the fact that Wang and co-workers (2011) developed the CAPS2 marker based on an American melon inbred line (Wang et al., 2011), one possibility for this discordance is that the resistance allele in our material is different from the allele in that line. The LRR domain of NBS-LRR proteins is under diversifying selection (Ellis at al., 2006), and thus, it is quite possible to have variation in the LRR domain of resistance alleles of *Fom-2*.

Conclusion

Here, we reported sources of resistance allele for *Fom-2* gene in two landraces of muskmelon in Khorasane-e-Razavi, Iran. These landraces can be used in breeding projects to enhance Fusraium resistance in bred cultivars as well as the landraces. The molecular markers developed for resistance to Fusarium can be used for high-throughput screening of landraces.

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Conflict of interest

The authors declare no conflict of interest for this study.

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