Euphytica
DOI 10.1007/s10681-015-1420-5

Molecular characterization of *Fom-1* gene and development of functional markers for molecular breeding of resistance to Fusarium race 2 in melon

A. Oumouloud · M. El Otmani · J. M. Álvarez

Received: 29 December 2014 / Accepted: 10 March 2015

Abstract Melon Fusarium wilt disease caused by the soil-borne pathogen *Fusarium oxysporum* f. sp. *melonis* (*Fom*) is one of the most devastating melon diseases worldwide. Recently, the *Fom-1* gene responsible for resistance against *Fom* races 0 and 2 was cloned. In this study we amplified, cloned and sequenced full genomic DNA and cDNA of *Fom-1* from several melon resistant and susceptible accessions using three pairs of primers designed within this gene. Sequence analyses showed that this gene contains four exons interrupted by three introns. The comparative sequence analysis of the cloned cDNA amplicons from resistant and susceptible genotypes revealed eight nucleotide substitutions, within *Fom-1* coding regions, among which four were non-synonymous. RT-PCR revealed that the *Fom-1* expression is induced by *Fom* race 2 inoculation. The *Fom-1* predicted protein (FOM-1) exhibits a tripartite modular structure composed of an N-terminal TIR domain, a central NB–ARC domain and a C-terminal LRR domain. FOM-1 from resistant melon accessions share four amino acid differences relative to the FOM-1 protein in susceptible ones. Two amino acid substitutions N56K and R103H were located at the FOM-1 TIR domain and the substitution E385K in the NB–ARC domain. Based on single nucleotide polymorphisms within the coding region of the *Fom-1* locus, we have generated two CAPS markers, Fom-1R and Fom-1S. Results from screening various melon accessions clearly demonstrated the usefulness of both functional CAPS markers in the marker-assisted selection for melon breeding programs.

Keywords Disease · CAPS · SNP · TIR–NBS–LRR · Marker-assisted selection

Introduction

Melon Fusarium wilt is a widespread and serious melon disease caused by the soilborne fungus *Fusarium oxysporum* f. sp. *melonis* Snyder and Hans (*Fom*). Four races of *Fom* have been identified and named 0, 1, 2, and 1, 2 (Risser et al. 1976). The only available practical and efficient control is soil fumigation. It is an expensive and with harmful environmental effects. Genetic resistance might be the most effective alternative method to control this melon disease. So far, two race specific resistant genes, *Fom-1* and *Fom-2* have been identified in melon lines 'Doublon' and 'CM17187', respectively (Risser et al. 1976). *Fom-1* confers resistance to races 0 and 2, whereas *Fom-2* determines resistance to races 0 and 1.

A. Oumouloud (✉) · M. El Otmani
Institut Agronomique et Vétérinaire Hassan II, P.O. Box 1123, Agadir, 80 000 Agadir, Morocco
e-mail: ali.oumouloud@gmail.com

J. M. Álvarez
Centro de Investigación y Tecnología Agroalimentaria de Aragón, Apartado 727, 50080 Zaragoza, Spain

Published online: 17 March 2015

To date, these genes have been extensively used in melon breeding and were already introduced to the majority of modern melon cultivars (Oumouloud et al. 2013). The *Fom-2* gene has been mapped into linkage group XI of melon map and isolated by a map based cloning strategy (Joobeur et al. 2004). The information generated from the *Fom-2* LRR region sequences allowed systematic development of functional markers that were developed based on the nucleotide polymorphisms detected between the susceptible and resistant *Fom-2* alleles (Wang et al. 2011; Oumouloud et al. 2012).

The *Fom-1* gene has been mapped at the distal part of the linkage group IX of melon map. Using RAPD or AFLP markers and the 'bulked segregant analysis' (BSA) method several molecular markers linked to this gene were identified (Brotman et al. 2005; Oumouloud et al. 2008, 2009; Tezuka et al. 2009). In addition, the technique based on the cloning of resistance gene homologues (RGH) has also been described as an approach for tagging resistance genes since they are often arranged in clusters in the plant genome (Yu et al. 1996; Leister et al. 1998). Using this method, Garcia-Mas et al. (2001) have identified the first melon resistance gene homologue (MRGH21) linked to *Fom-1*. Subsequent studies established that the melon genomic region harboring *Fom-1* contains a cluster of eight melon RGHs, and the BAC (BAC 31O16) encompassing this RGHs was sequenced and characterized (Van Leeuwen et al. 2005). Based on the BAC 31O16 sequence Tezuka et al. (2011) developed more DNA markers linked to *Fom-1*, nevertheless, the authors enabled positional cloning of the locus, but they suggested that *Fom-1* mapped between C-MRGH13 and 62-CAPS markers, which delimits a 137.462 bp interval.

Nowadays, the usefulness of the described markers in determining the *Fom-1* genotype is somewhat limited since they do not separate all genotypes according to their resistance phenotype; instead, it seems that each marker will have application only in a specific melon genetic background. More recently, Brotman et al. (2013) isolated the *Fom-1* gene by a map-based cloning strategy in a population derived from the backcross ('Védrantais' × PI 414723) × PI 414723. They have established that MRGH-9 corresponds to gene *Fom-1*, but they did not achieve the full characterization of this gene, even if many details have yet to be clarified.

In this study, we also intend to complete the characterization of Fom-1; therefore, we cloned the complete *Fom-1* cDNA in melon lines resistant and susceptible to *Fom* race 2, and revealed detailed features characteristic of the protein of this gene. Our findings provide new insight to the structure and expression of the *Fom-1* gene. Also, we present, for the first time, the functional molecular markers for *Fom-1*-mediated resistance against *Fom* races 0 and 2.

Materials and methods

Plant and fungal materials

The melon material used in this study consisted of 11 accessions and one F_1 hybrid genotype resistant to *Fom* race 2, and twelve accessions susceptible to this race (Table 1). This material was provided by the Vegetable Germplasm Bank of Zaragoza, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Zaragoza, Spain. All accessions were inoculated with the isolate *Fom-8701*, belonging to *Fom* race 2, using the inoculation method described in Oumouloud et al. (2010). For each accession, 12 plants were tested. The lines Charentais-Fom1 (Char-Fom1) and Charentais-Fom2 (Char-Fom2) were used as resistant and susceptible controls to *Fom* race 2, respectively. Moreover, 12 seedlings per accession remained as un-inoculated controls.

DNA extraction

Fresh leaf tissue (2–3 g) at the one- to two- true leaf stages was collected from each accession, ground in liquid nitrogen and genomic DNA was extracted following the method of Doyle and Doyle (1987) with minor modifications as previously described in Oumouloud et al. (2008). DNA yield was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For PCR technique, the DNA concentration was adjusted to 10 ng/ml with Millipore water.

Total RNA isolation and cDNA synthesis

Hypocotyls were collected from melon young plants and immediately frozen in liquid nitrogen. Tissues were ground in liquid nitrogen using a mortar and

Table 1 Melon (*Cucumis melo* L.) accessions used for the molecular analysis: their origin, horticultural type, and reaction to *Fom* race 2

N°	Genotype	Origin	Horticultural type	Reaction to *Fom* race 2[x]
01	Charentais-Fom1	France	*cantalupensis*	R
02	Charentais-Fom2	France	*cantalupensis*	S
03	Tortuga	Spain	*cantalupensis*	R
04	Charentais-T (Ch-T)	France	*cantalupensis*	S
05	Perlia-FR	France	*cantalupensis*	R
06	Top mark	USA	*cantalupensis*	S
07	Doublon	France	*cantalupensis*	R
08	WI-998	US	*cantalupensis*	S
09	Piel de Sapo Monoico	Spain	*inodorus*	R
10	Piel de Sapo	Spain	*inodorus*	S
11	Amarillo Cáscara Pinta	Spain	*inodorus*	R
12	Amarillo oval	Spain	*inodorus*	S
13	Amarillo manchado	Spain	*inodorus*	R
14	Amarillo exportación	Spain	*inodorus*	S
15	Banda de Godoy	Spain	*inodorus*	R
16	C-181	Japan	*inodorus*	S
17	C-160	Russia	*inodorus*	R
18	Kirkagaç	Turkey	*inodorus*	S
19	Cum-334	Tajikistan	*inodorus*	R
20	Bola de Oro	Spain	*inodorus*	S
21	PI-124111	India	*momordica*	R
22	Ananas	Kenya	*reticulatus*	S
23	Cum-334 × Ch-T	–	–	R
24	Galia	France	*reticulatus*	S

x R and S indicate Fusarium wilt resistant and susceptible phenotypes respectively

pestle, then 100 mg of the powder were transferred to an Eppendorf tube with Trizol_Reagent (Life Technologies, Carlsbad, CA, USA) and RNA was extracted according to the manufacturer's protocol with the following modifications. For precipitation, only half of the recommended volume of isopropanol was used, with the other half being replaced with salt solution (0.8 M sodium citrate, 1.2 M NaCl). Pellets were dissolved in 30 µl RNase-free water and incubated at 65 °C for 3–5 min before the concentration of total RNA was quantified at 260 nm using the NanoDrop spectrophotometer (NanoDrop ND-100, NanoDrop Technologies, Wilmington, DE, USA). The quality of total RNA was assessed using 1.2 % (w/v) agarose gel electrophoresis, and samples showing a good RNA quality were selected and stored at −80 °C until further analysis.

One microgram of total RNA was DNase-treated at room temperature for 15 min in 10 µL reactants containing 1 U of DNase I (amplification grade, Life technologies). Then, EDTA was added to a final concentration of 2.5 mM and the DNase was inactivated at 65 °C for 15 min. First strand cDNA was synthesized from 1 µg of the total RNA by RT–PCR using oligo$(dT)_{20}$ primer and SuperScriptfi III First-Strand Kit (http://www.lifetechnologies.com) according to the manufacturer's instructions.

Primer design and PCR analysis

The MRGH-9 sequence (Genebank accession number AAU04764.1) was used as a reference to develop primers targeting the complete *Fom-1* sequence (Fig. 1). For this, gene-specific primer pairs, RG9-1F/R, RG9-2F/R and RG9-3F/R were designed (Table 2) using the primer design tool Primer3 software (Rozen and Skaletsky 2000). To prevent amplification of residual genomic DNA, at least one primer of each pair was positioned across an exon–exon boundary. DNA or cDNA amplifications were

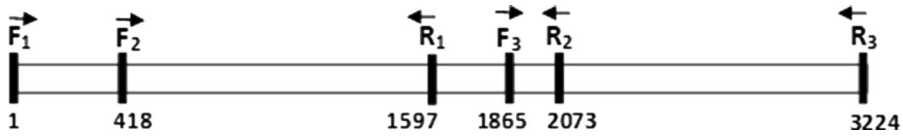

Fig. 1 Schematic representation of the three amplified DNA genomic regions of the *Fom-1* gene from *Cucumis melo*; *arrows* indicate the annealing positions of the primers used for PCR analyses

Table 2 Sequences of primers used for the genomic DNA and cDNA amplification

Marker name	Primer sequences
RG9-1F	5′-ATGAGTTTTGATAGTTTCATAAG-3′
RG9-1R	5′-TTACCAATTCCGCCCATCC-3′
RG9-2F	5′-CAATGGAGGAGGGCTTTCA-3′
RG9-2R	5′-TAACCCTTTCTACAATCTCCT-3′
RG9-3F	5′-TTGGTTTGGTTCAGGGTCC-3′
RG9-3R	5′-CTATTTACCATAATGAAAGTTAC-3′
Fom-1_{568}F	5′-ATGAGTTTTGATAGTTTCATAAG-3′
Fom-1_{568}R	5′-GAACACTCCCTTAGATACTT-3′

performed by PCR according to Oumouloud et al. (2008) except that the annealing temperature was optimized for each specific pair of primers. Amplification products were separated by electrophoresis on 2 % agarose gels in 1 × TAE (90 mM Tris–Acetate and 2 mM EDTA, pH 8.0), stained with ethidium bromide at 50 ng/μL and visualized under UV light with an image analysis system (GelDoc2000, Bio-Rad, Hercules, CA, USA).

Cloning, nucleotide sequencing and sequence analysis

Genomic and cDNA amplified products were cloned into the pGEM-T plasmid vector (Promega, Madison, WI, USA). JM109 High Efficiency Competent Cells (Promega) were transformed with these plasmid vectors (Sambrook et al. 1989), and the plasmids were purified using QIAprep kits (Qiagen). Sequencing of the cloned fragments was conducted by the Secugen S.L., (Centro de Investigaciones Biológicas, CSIC, Madrid, Spain). The cloning and sequencing experiments were conducted independently three times for all amplified fragments. The sequence of the MRGH9 encompassing *Fom-1* gene was analyzed with the gene prediction programs GENSCAN (http://genes.mit.edu/GENSCAN.html) and FGENESH (http://www.softberry.com). Sequence analysis and alignments were carried out using the online version of CLUSTALW (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and SNPs were developed into CAPS markers. Analysis of *Fom-1* predicted protein homology was performed using Pfam analysis (http://pfam.sanger.ac.uk/).

Results

Sequencing of genomic DNA and cDNA of melon *Fom-1* gene

Using the three primer pairs RG9-1F/R, RG9-2F/R and RG9-3F/R, DNA fragments with the size of 1597-, 1656-, and 1360-bp were amplified respectively by PCR from the four melon lines Char-Fom1, Char-Fom2, 'Piel de Sapo Monoico' (PSM) and 'Piel de Sapo' (PS). Whereas, the fragments amplified from cDNA were 677-, 736-, and 1207-bp respectively. In the Fig. 2, only the amplification results from Char-Fom1 and Char-Fom2 are presented. For each line, the PCR products amplified both from DNA and cDNA were cloned, sequenced, and the overlapping sequences between the PCR products were combined and fused to generate a full-length DNA and cDNA sequence. As expected, **t**he full-length of genomic DNA and cDNA sequences were 3.224- and 2151-pb respectively. These results revealed that the genomic amplification fragments were much larger than those expected on the basis of the cDNA template, indicating the presence of introns in the *Fom-1* gene.

Structural and expression analysis of the melon *Fom-1* gene

Comparative analysis of the cDNA and the genomic sequence permitted us to characterize four exons interrupted by three introns (Fig. 3). Exon sequence lengths were: exon 1: 477-bp; exon 2: 1093-bp; exon 3: 123-bp; and exon 4: 264-bp (including the stop

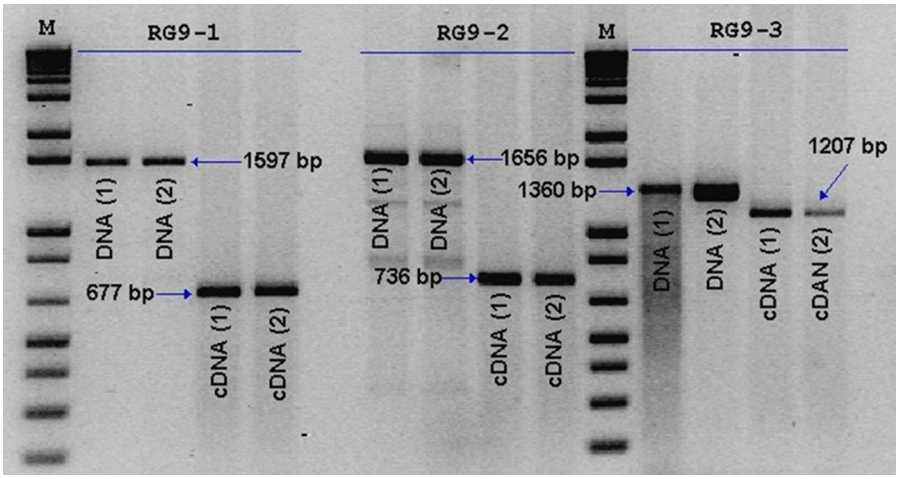

Fig. 2 Agarose gel electrophoresis of PCR products obtained from genomic DNA and cDNA in Charentais-Fom1 (1) and Charentais-Fom2 (2) lines, amplified with primer pairs RG9-1F/R, RG9-2F/R, and RG9-3F/R. *M* molecular size marker 1 kb plus ladder

codon). The sizes of the introns were determined by sequencing both strands of DNA. Conserved splicing sites (GT and AG) were present at the three-intron/exon junctions. The positions of the exons and introns of the *Fom-1* gene were confirmed by using the programs GENSCAN and FGENESH for exon–intron prediction.

RT-PCR was used to detect *Fom-1* gene expression following infection by *Fom* race 2. Total RNA was isolated from hypocotyl tissue of melon seedlings from the resistant lines Char-Fom1 and PSM, and the susceptible ones Char-Fom2 and PS, at 4-days after inoculation with *Fom* race 2 and from un-inoculated seedlings. The RT-PCR reactions were performed using the three primer pairs RG9-1F/R, RG9-2F/R and RG9-3F/R. For each pair primer, the RT-PCR allowed the amplification of a fragment with the expected size in the inoculated lines. However, no amplification products were detected from cDNA template derived from un-inoculated lines. (In the Fig. 4, only the amplification products from cDNA of Char-Fom1 and Char-Fom2 are presented). These results suggested that the *Fom-1* gene was not expressed or was severely reduced in un-inoculated plants and its expression was up-regulated during a post-inoculation period.

Predicted structure of the *Fom-1* gene product and sequence comparison between resistant and susceptible alleles

Translation of the 2151-bp *Fom-1* gene-coding region resulted in a predicted protein (FOM-1) of 716 amino acids shown in Fig. 5, with an expected size of 83.05-kD and a Pi of 6.892. A pfam analysis revealed that FOM-1 appears to have characteristic features from the proteins encoded by resistance genes (Hammond-Kosack and Jones 1997). FOM-1 exhibits a tripartite modular structure composed of an N-terminal TIR domain (amino acids 5–147), a central NB–ARC domain (amino acids 195–459), and a C-terminal LRR domain (amino acids 599–618).

Multiple alignments of *Fom-1* coding sequence cloned from melon resistant accessions (Char-Fom1, PSM, Tortuga, Perlita-FR, Doublon, Amarillo Cáscara

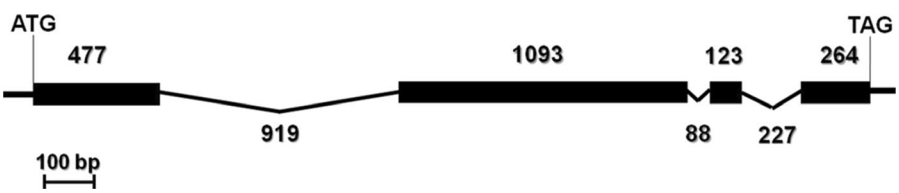

Fig. 3 Schematic diagram of the *Fom-1* gene coding sequence. Exons are indicated by *black rectangles*. Introns are indicated by *broken lines*. The *numbers* indicate the size of introns and exons in bp

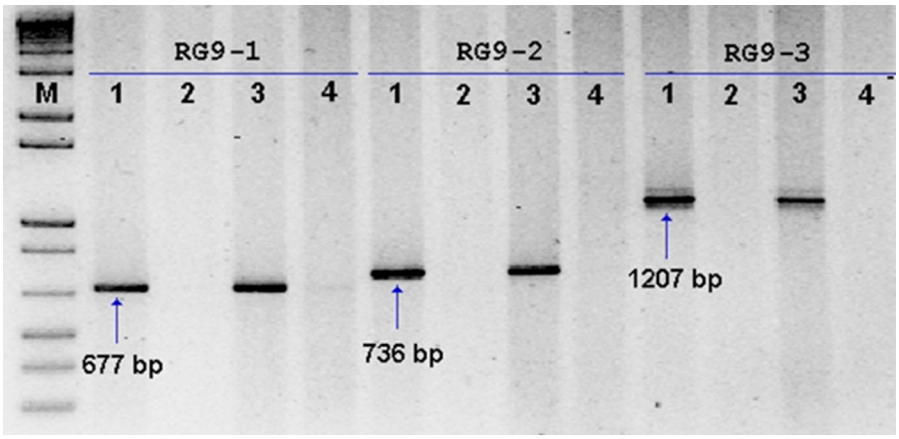

Fig. 4 Agarose gel electrophoresis of PCR products amplified with primer pairs RG9-1F/R, RG9-2F/R, and RG9-3F/R in cDNA isolated from un-inoculated and inoculated melon lines Charentais-Fom1 and Charentais-Fom2 with *Fom* race 2. *M* molecular size marker 1 kb plus ladder, *1* inoculated Charentais-Fom1, *2* un-inoculated Charentais-Fom1, *3* inoculated Charentais Fom2, *4* un-inoculated Charentais-Fom2

Pinta, Amarillo Manchado, Banda de Godoy, C-160, Cum-334, and PI-124111) revealing that the sequence of *Fom-1* is conserved between these 12 unrelated, resistant accessions and is identical to the previously characterized Védrantais resistance allele (Brotman et al. 2013). However, when compared with the sequence cloned from the susceptible accessions (Char-Fom2, PS, 'Amarillo Exportación', 'Ananas', and 'Galia') eight nucleotide substitutions within *Fom-1* coding regions were observed. In fact, the *Fom-1* resistant allele carried the following substitutions relative to the susceptible allele *fom1*: C–A at position 168-bp (abbreviated C168A), G300C, G308A, T378C, A468G, G474A and T478A all situated at exon-1, and G1153A located at exon-2. Only the substitutions C168A, G308A, T378C and G1153A resulted in the amino acid changes N56 K, R103H, C160S and E385K respectively (Fig. 5). When the nucleotide sequences of *Fom-1* resistant and susceptible alleles were compared, the ratio between non-synonymous (Ka) and synonymous nucleotide substitution rates was much lower than 1 (Ka/Ks = 0.2386) supporting the hypothesis that this gene is not under selection pressure.

Development and application of allele-specific PCR markers

From the eight SNPs identified within *Fom-1* coding regions, only the C168A and G308A substitutions generate known enzyme restriction sites allowing the development of two allele-specific CAPS markers, designated Fom1-R and Fom1-S (Table 3). To simplify the restriction digest results, a 568-bp fragment was amplified from the genomic DNA of resistant and susceptible genotypes using the pair primers $Fom1_{568F}$/$Fom1_{568R}$ (Table 2) flanking these two SNPs region. The Fom1-R CAPS marker was developed based on the C168A substitution that conditioned the presence (resistant) or absence (susceptible) of the *BspCNI* restriction endonuclease CTCAG(N)10 site. Thereafter, BspCNI digestion of 568-bp PCR product showed patterns of 182- and 386-bp fragments in the resistant homozygous genotypes, three fragments 182-, 386- and 568-bp in the resistant heterozygous genotypes and uncut 568-bp fragment in the susceptible ones (Fig. 6A). Similarly, the Fom1-S CAPS marker was obtained based on the G308A substitutions that eliminate *BspHI* restriction endonuclease T/CATGA site in resistant material. The *BspHI* cleaved the 568-bp amplified product into 262- and 306-bp fragments in the susceptible genotypes, 262-, 306- and 568-bp fragments in the resistant heterozygous genotypes, and an uncut fragment of 568-bp in the homozygous resistant ones (Fig. 6B). Validation using a collection of 23 accessions and one hybrid cross showed that both CAPS are 100 % predictive of the presence or absence of the *Fom-1* gene. Among the 24 tested genotypes, 12 were identified as being resistant to *Fom* races 0 and 2, including 10 homozygous and two heterozygous genotypes (Fig. 6).

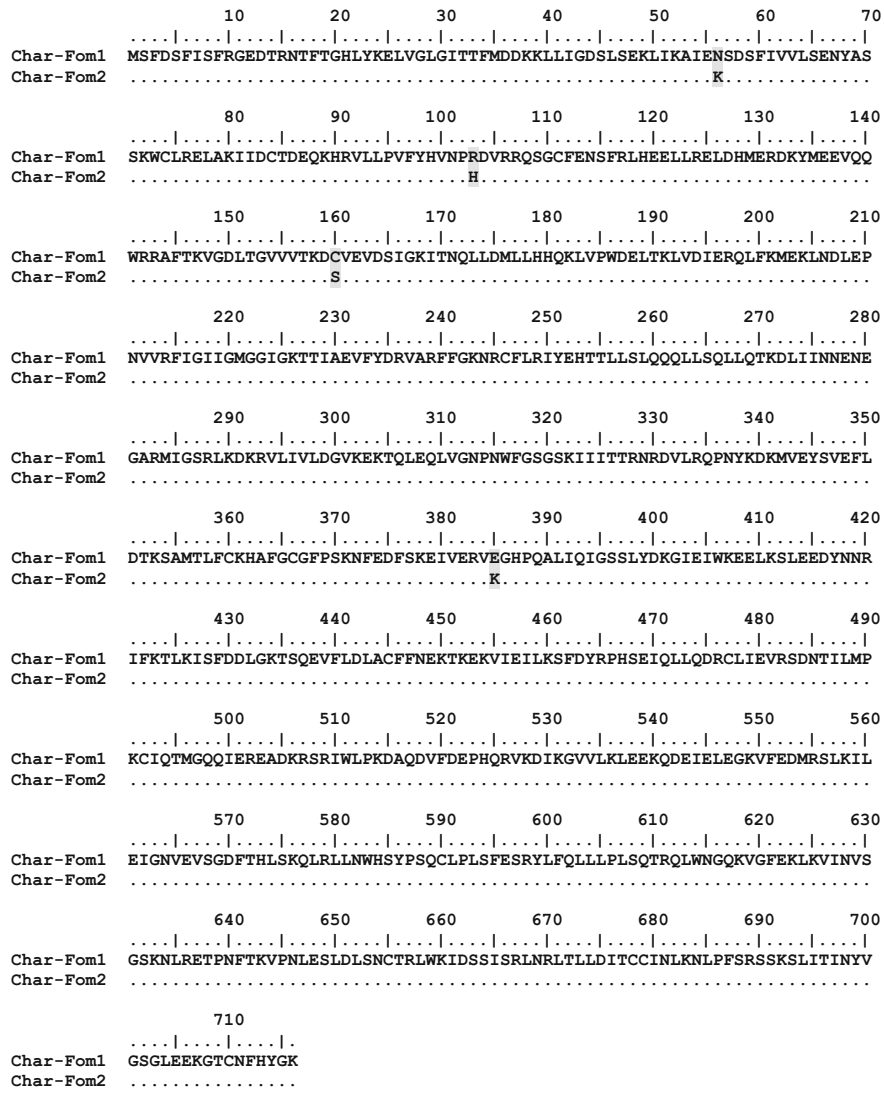

Fig. 5 Comparison of the predicted amino acid sequences of the FOM-1 protein from resistant and susceptible melon lines. The *boldfaces* show amino acid differences between the resistant and susceptible lines

Discussion

Fom-1 is a single dominant resistance gene that is effective against *Fom* races 0 and 2. In a previous report, Brotman et al. (2013) identified the MRGH9 as a candidate gene for the *Fom-1* locus, through a map-based cloning strategy, and in the melon genome annotation this gene was designated Melo3c022146. In the present study, the MRGH9 sequence was used to develop some primers that target the full genomic DNA; and cDNA of the *Fom-1* gene were amplified, cloned and sequenced from resistant and susceptible melon accessions. The optimization of PCR assays all resulted in a single product with the desired length and without non-specific band suggesting the accuracy of the three primer pairs RG9-1F/R, RG9-2F/R and RG9-3F/R in isolating this *R*-gene. By overlapping sequences, we established that the full-length of genomic DNA and cDNA of *Fom-1* were 3224- and 2151-bp, respectively. This fits very well with the results on other characterized R-genes, in most cases the coding sequence is about 3-kb (Meyers et al. 2003; Jupe et al. 2012). All genomic amplification fragments were much larger than expected on the basis of the cDNA template, indicating the presence of introns in the *Fom-1* gene. Sequence analyses showed that this gene contains four exons interrupted by three introns. All exon–intron junctions obeyed the universal GT/

Table 3 Primers and restriction enzymes used for *Fom-1* allele specific CAPS markers in melon

Marker name	PCR product size (pb)	Restriction enzyme for SNPs detection	Fragment size after digestion (pb)	Expected genotype
Fom1-R	568	*BspCNI* (CTCAG(N)10)	182 + 386	*Fom-1/Fom-1*
			182 + 386 + 568	*Fom-1/fom-1*
			568	*Fom-1/fom-1*
Fom1-S	568	*BspHI* (T/CATGA)	262 + 306	*Fom-1/fom-1*
			262 + 306 + 568	*Fom-1/fom-1*
			568	*Fom-1/Fom-1*

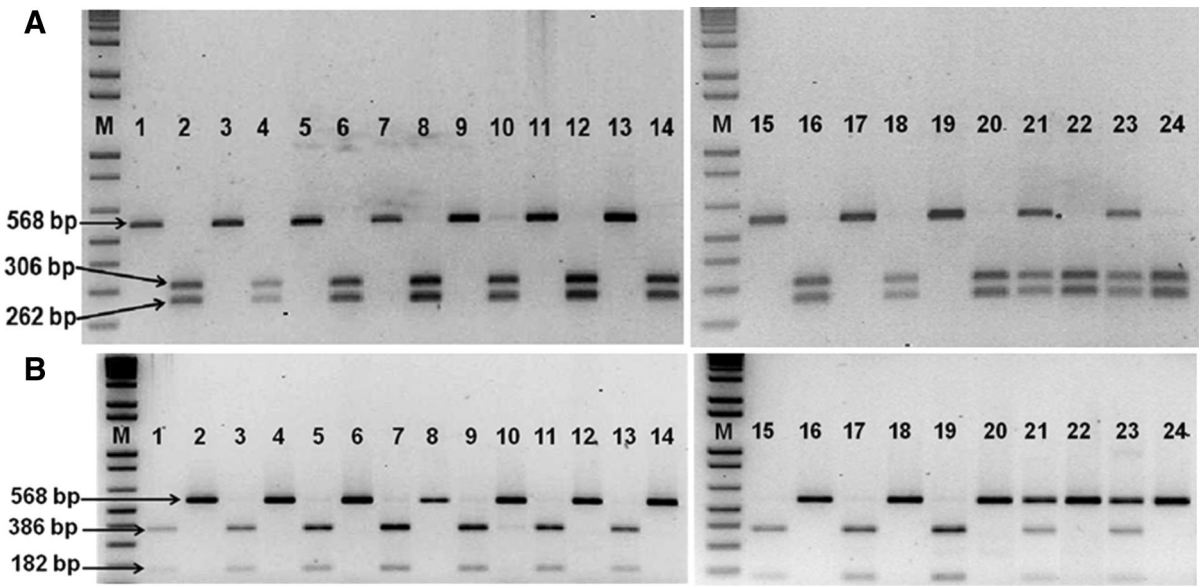

Fig. 6 Application of two allele-specific CAPS markers for resistant and susceptible alleles at the *Fom-1* locus. **A** DNA analysis of Fom-1R CAPS marker. *BspCNI* digest generates: 182- and 386-bp products for resistant genotype *Fom1/Fom1*; 182-, 386- and 568-bp products for resistant genotype *Fom1/*fom1; and 568-bp products for susceptible genotype *fom1/fom1*. **B** DNA analysis of Fom-1S CAPS marker. *BspHI* digest generates: 262- and 306-bp products for susceptible genotype *fom1/fom1*; 262-, 306- and 568-bp products for resistant genotype *Fom1/*fom1; and 568-bp products for resistant genotype *Fom1/Fom1*. PCR products were separated by electrophoresis in a 2 % agarose gel. bp base pairs; M 1 kb DNA ladder; lines 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 resistant genotypes; lines 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 susceptible genotypes (Table 1)

AG rule of splicing and fitted quite well to the consensus sequence for splice donor and acceptor sites for plant genes (Simpson and Filipowicz 1996).

The FOM-1 predicted protein contained the different features of the TIR class of NBS–LRR R-proteins (TNL). Compared to the FOM-2 protein, encoded by *Fom-2* gene mediating resistance to *Fom* races 0 and 1, FOM-1 showed only 17 % identity (E value = e − 148; 30 % positives), revealing an independent evolution of these two race specific resistant genes in melon. More recently, from the analysis of the melon genome sequence, 21 TNL *R*-genes were identified; seven were clustered in interval of less than 135-kb and located at the region harboring the *Fom-1* gene, whereas 13 *R*-genes spanning a region of 570-kb are located in the same region of the melon *Vat* resistance gene (Garcia-Mas et al. 2012). Furthermore, the distribution of introns over the length of the *Fom-1* coding sequence revealed that these introns separating distinct protein domains. Introns separating distinct protein domains of *R*-genes are quite common in dicotyledonous species. In fact the *Fom-1* first intron,

separating the encoded TIR and NBS domains, also was present in three flax genes, *L6*, *M*, and *P* (Lawrence et al. 1995; Anderson et al. 1997; Dodds et al. 2001), and in the tobacco *N* gene (Whitham et al. 1994). The second intron, after the NBS domain, was conserved in the tobacco *N*, flax *L6* and *M* genes (Dodds et al. 2001). The third intron, at the end of the encoded LRR domains was present in all of the flax and tobacco genes and was important for alternative splicing (Anderson et al. 1997; Dinesh-Kumar and Baker 2000).

Furthermore, sequence analysis revealed that the sequence of *Fom-1* in the 11 unrelated resistant accessions is identical to the previously characterized Védrantais resistance allele (Brotman et al. 2013). This result indicates that *Fom-1* may belong to an *R*-gene group that shows low levels of polymorphism. Analogous findings have been reported for other *R*-genes including *Pi-d2* (Chen et al. 2006), *RB* (Song et al. 2003) and *Xa26/Xa3* (Xiang et al. 2006), where only two alleles, a resistant and a susceptible allele, have been identified. FOM-1 protein from resistant melon accessions share the common four amino acid differences relative to FOM-1 protein in susceptible ones. Two amino acid substitutions N56K and R103H were located at the FOM-1 TIR domain and the substitution E385K in the NB–ARC domain. A large number of mutations in both the TIR and NB–ARC domains have been found to inactivate the plant R proteins (Lukasik-Shreepaathy et al. 2012; van Ooijen et al. 2008a, b; Wladimir et al. 2006) implying that the mutated residues are important for R protein function. Recently, the crystal structure of the TIR domain from R protein *L6* from flax (*Linum usitatissimum*) that confers resistance to the flax rust phytopathogenic fungus (*Melampsora lini*), was determined and this structure, combined with functional studies, demonstrated that the TIR-domain is a requirement for function of the R protein *L6* (Bernoux et al. 2011). In addition, specific point mutations in the NB–ARC domain were found to confer either auto-activity or loss-of-function in full-length tomato *Mi-1.2* gene, which confers resistance against nematodes, whiteflies, psyllids and aphids (van Ooijen et al. 2008a, b).

The RT-PCR technique revealed that the *Fom-1* gene was not expressed, or its expression was severely reduced in the un-inoculated plants. This result is consistent with the findings of Wang et al. (2014) who showed, through quantitative real time PCR, low level of *Fom-2* transcript in the un-inoculated plants. A similar pattern has been observed in a majority of cloned *R*-genes (Mes et al. 2000; Song et al. 2003; Tan et al. 2007; Kar et al. 2013). Moreover, RT-PCR revealed specific transcript expression of *Fom-1* gene either in resistant and susceptible material after *Fom* infection. We considered that the susceptible allele is always expressed, its expression is not a response to the presence or not of the fungus, but the incompatible (resistant) reaction is only induced when the *Fom-1* resistant gene is expressed. The same type of expression was found for the *Ve* gene in root, stem, and leaf tissues from susceptible and resistant tomato cultivars 'Money Maker' and 'Motelle', respectively after inoculation with race 1 of *Verticillium dahliae* (Fradin et al. 2009). So far, functional analysis of *Fom-1* gene in resistant and susceptible melon plants remains to be investigated.

Until now, marker-assisted selection for *Fom-1* has involved markers that are only linked to this resistance gene within the genetic background they were developed in, and which risk being separated from the trait by recombination (Brotman et al. 2005; Oumouloud et al. 2008, 2009; Tezuka et al. 2009, 2011). Here we present two simple and efficient CAPS markers Fom1-R and Fom1-S based on single nucleotide polymorphisms within the coding region of the *Fom-1* locus encoding for resistance to *Fom* races 0 and 2. We demonstrate the utility of this marker system across 23 accessions and one hybrid representing several melon types from diverse origins that possess or lack the *Fom-1* gene. This is, to the best of our knowledge, the first report of a functional marker for *Fom-1*-mediated resistance to *Fom* races 0 and 2 in melon. It is evident that using these allele-specific CAPS markers for the *Fom-1* locus will reduce time, cost and considerable effort and will likely improve accuracy in introgressing this into susceptible melon germplasm.

Our study resulted in the isolation of the *Fom-1* locus from the resistant and susceptible melon lines via PCR with primers developed on the basis of the sequence of this gene, and provided insight about the *Fom-1* structure. The CAPS marker developed in this study should allow routine marker assisted selection for resistance against *Fom* races 0 and 2 in melon, although a crystal structure of TIR and NB–ARC FOM-1 domains is required to provide insight into the function of the conserved elements within these domains and to shed light on the molecular

mechanisms through the detected how mutations in these domains exert their effect.

References

Anderson PA, Lawrence GJ, Morrish BC, Ayliffe MA, Finnegan EJ, Ellis JG (1997) Inactivation of the flax rust resistance gene *M* associated with loss of a repeated unit within the leucine-rich repeat coding region. Plant Cell 9:641–651

Bernoux M, Ve T, Williams S, Warren C, Hatters D, Valkov E, Zhang X, Ellis JG, Kobe B, Dodds PN (2011) Structural and functional analysis of a plant resistance protein TIR domain reveals interfaces for self-association, signaling, and autoregulation. Cell Host Microbe 9:200–211

Brotman Y, Kovalski I, Dogimont C, Pitrat M, Portnoy V, Perl-Treves R (2005) Molecular markers linked to papaya ring spot virus resistance and *Fusarium* race 2 resistance in melon. Theor Appl Genet 110:337–345

Brotman Y, Normantovich M, Goldenberg Z, Zvirin Z, Kovalski I, Stovbun N, Donniger T, Bolger AM, Troadec C, Bendahmane A, Cohen R, Katzir N, Pitrat M, Dogimont C, Perl-Treves R (2013) Dual resistance of melon to *Fusarium oxysporum* races 0 and 2 and to *Papaya ring-spot virus* is controlled by a pair of head-to-head oriented NB–LRR genes of unusual architecture. Mol Plant 6:235–238

Chen X, Shang J, Chen D, Lei C, Zou Y, Zhai W, Liu G, Xu J, Ling Z, Cao G, Ma B, Wang Y, Zhao X, Li S, Zhu L (2006) A B-lectin receptor kinase gene conferring rice blast resistance. Plant J 46:794–804

Dinesh-Kumar SP, Baker BJ (2000) Alternatively spliced *N* resistance gene transcripts: their possible role in tobacco mosaic virus resistance. Proc Natl Acad Sci USA 97:1908–1913

Dodds PN, Lawrence GJ, Ellis JG (2001) Six amino acid changes confined to the leucine-rich repeat beta-strand/beta-turn motif determine the difference between the *P* and *P2* rust resistance specificities in flax. Plant Cell 13:163–178

Doyle JJ, Doyle JL (1987) A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Fradin EF, Zhang Z, Ayala JCJ, Castroverde CDM, Nazar RN, Robb J, Liu CM, Thomma BPHJ (2009) Genetic dissection of *Verticillium* wilt resistance mediated by tomato *Ve1*. Plant Physiol 150:320–332

Garcia-Mas J, Leeuwen HV, Monfort A, Carmen de Vicente M, Puigdomenech P, Arus P (2001) Cloning and mapping of resistance gene homologues in melon. Plant Sci 161:165–172

Garcia-Mas J et al. (2012) The genome of melon (*Cucumis melo* L.). Proc Natl Acad Sci USA 109:11872–11877

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48:575–607

Joobeur T, King JJ, Nolin SJ, Thomas CE, Dean RA (2004) The *Fusarium* wilt resistance *locus Fom-2* of melon contains a single resistance gene with complex features. Plant J 39:283–297

Jupe F, Pritchard L, Etherington GJ, Mackenzie K, Cock PJ, Wright F, Kumar Sharma S, Bolser D, Bryan GJ, Jones JD, Hein I (2012) Identification and localization of the NB–LRR gene family within the potato genome. BMC Genom 13:75

Kar B, Nanda S, Nayak PK, Nayak S, Joshi RK (2013) Molecular characterization and functional analysis of *CzR1*, a coiledcoil-nucleotide-binding-site-leucine-rich repeat *R*-gene from *Curcuma zedoaria* Loeb. that confers resistance to *Pythium aphanidermatum*. Physiol Mol Plant Pathol 83:59–68

Lawrence GJ, Finnegan EJ, Ayliffe MA, Ellis JG (1995) The *L6* gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and tobacco viral resistance gene *N*. Plant Cell 7:1195–1206

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefert P (1998) Rapid reorganization of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95:370–375

Lukasik-Shreepaathy E, Slootweg E, Richter H, Goverse A, Cornelissen BJC, Takken FLW (2012) Dual regulatory roles of the extended N terminus for Activation of the tomato *Mi-1.2* resistance protein. Mol Plant-Microbe Interact 25:1045–1057

Mes J, Van Doorn A, Wijbrandi J, Simons G, Cornelissen B, Haring M (2000) Expression of the *Fusarium* resistance gene *I-2* co-localizes with the site of fungal containment. Plant J 23:183–193

Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS–LRR-encoding genes in *Arabidopsis*. Plant Cell 15:809–834

Oumouloud A, Arnedo-Andres MS, Gonzalez-Torres R, Álvarez JM (2008) Development of molecular markers linked to the *Fom-1* locus for resistance to Fusarium race 2 in melon. Euphytica 164:347–356

Oumouloud A, Arnedo-Andres MS, González-Torres R, Álvarez JM (2009) Morphological and molecular characterization of melon accessions resistant to Fusarium wilts. Euphytica 169:69–79

Oumouloud A, Arnedo-Andrés MS, González-Torres R, Álvarez JM (2010) Inheritance of resistance to *Fusarium oxysporum* f. sp. *melonis* races 0 and 2 in melon accession Tortuga. Euphytica 176:183–189

Oumouloud A, Mokhtari M, Chikh-Rouhou H, Arnedo-Andrés MS, González-Torres R, Álvarez JM (2012) Characterization of the Fusarium wilt resistance *Fom-2* gene in melon. Mol Breed 30:325–334

Oumouloud A, El-Otmani M, Chikh-Rouhou H, Garcés-Claver A, González-Torres R, Perl-Treves R, Alvarez JM (2013) Breeding melon for resistance to Fusarium wilt: recent developments. Euphytica 192:155–169

Risser G, Banihashimi Z, Davis DW (1976) A proposed nomenclature of *Fusarium oxysporum* f. sp. *melonis* races and résistance genes in *Cucumis melo*. Phytopathology 66:1105–1106

Rozen S, Skaletsky H (2000) Primer3 on the www for general users and for biologist programmers. In: Krawetz SA, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology, vol 132. Humana Press, Totowa, pp 365–386

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Press, Plainview

Simpson GG, Filipowicz W (1996) Splicing of precursors to mRNA in higher plants: mechanism, regulation, and subnuclear organization of the spliceosomal machinery. Plant Mol Biol 32:1–41

Song J, Bradeen JM, Naess SK, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang H, Austin-Phillips S, Buell CR, Helgeson JP, Jiang J (2003) Gene *RB* cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci USA 100:9128–9133

Tan X, Meyers BC, Kozik A, West M, Morgante M, Clair DA, Bent AF, Michelmore RW (2007) Global expression analysis of nucleotide binding site-leucine rich repeat-encoding and related genes in *Arabidopsis*. BMC Plant Biol 7:56

Tezuka T, Waki K, Yashiro K, Kuzuya M, Ishikawa T, Takatsu Y, Miyagi M (2009) Construction of a linkage map and identification of DNA markers linked to *Fom-1*, a gene conferring resistance to *Fusarium oxysporum* f.sp. *melonis* race 2 in melon. Euphytica 168:177–188

Tezuka T, Waki K, Kuzuya M, Ishikawa T, Takatsu Y, Miyagi M (2011) Development of new DNA markers linked to the Fusarium wilt resistance locus *Fom-1* in melon. Plant Breed 130:261–267

Van Leeuwen H, Garcia-Mas J, Coca M, Puigdoménech P, Monfort A (2005) Analysis of the melon genome in regions encompassing TIR–NBS–LRR resistance genes. Mol Gen Genomics 273:240–251

Van Ooijen G, Mayr G, Albrecht M, Cornelissen BJ, Takken FL (2008a) Transcomplementation, but not physical association of the CC–NB–ARC and LRR domains of tomato R protein *Mi-1.2* is altered by mutations in the ARC2 subdomain. Mol Plant 1:401–410

Van Ooijen G, Mayr G, Kasiem MM, Albrecht M, Cornelissen BJ, Takken FL (2008b) Structure-function analysis of the NB–ARC domain of plant disease resistance proteins. J Exp Bot 59:1383–1397

Wang S, Yang J, Zhang M (2011) Developments of functional markers for *Fom-2*-mediated Fusarium wilt resistance based on single nucleotide polymorphism in melon (*Cucumis melo* L.). Mol Breed 27:385–393

Wang X, Xu B, Zhao L, Gao P, MA H, Luan F (2014) Expression analysis of Fusarium wilt resistance gene in melon by real-time quantitative PCR. Pak J Bot 46:713–717

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene *N*: similarity to toll and the interleukin-1 receptor. Cell 78:1101–1115

Wladimir IL, Tameling JH, Vossen MA, Lengauer T, Berden JA, Haring MA, Cornelissen BJC, Takken FLW (2006) Mutations in the NB–ARC Domain of *I-2* that impair ATP hydrolysis cause autoactivation. Plant Physiol 140:1233–1245

Xiang Y, Cao Y, Xu C, Li X, Wang S (2006) *Xa3*, conferring resistance for rice bacterial blight and encoding a receptor kinase-like protein, is the same as *Xa26*. Theor Appl Genet 113:1347–1355

Yu YG, Buss GR, Maroof MA (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93:11751–11756