Australasian Plant Pathol.
DOI 10.1007/s13313-016-0408-5

ORIGINAL PAPER

Tracking of the gene *Fom2* and study on the genetic diversity of NB-ARC domain in the number of resistant and sensitive melon cultivars against *Fusarium oxysporum* f. sp. *melonis* (race 1)

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Received: 21 November 2015 / Accepted: 16 March 2016

Abstract Vascular wilt caused by *Fusarium oxysporum* f. sp. *melonis* (*FOM*) is one of the most important diseases in melon. Four pathogenic races named: 0, 1, 2, and 1.2 have been identified in population of this fungus based on the pathogenicity pattern on differential varieties. The gene *Fom2* has been reported as a key gene for conferring resistance to the fungus race 1. The majority of Iranian melon cultivars are susceptible to race 1. Clarifying the occurrence pattern of *Fom*2 in the fungus race 1 in sensitive and resistant Iranian melon cultivars in comparison with two standard melon lines could be helpful for the investigation on the role of *Fom2* in the cultivars reaction. Since, the full sequence of *Fom2* from Melon and *I*2 from Tomato resistance sources were cloned; it has been shown that the LRR and NB-ARC domains in the coded proteins play critical roles in detection of Avr proteins and R proteins activation as well. Considering to the importance of NB-ARC domain and the lack of functional information in this region of the gene *Fom2*, this domain was selected for sequencing, identifying possible mutations and to study their possible relationship of mutations with the type of cultivars reaction against fungal races. Six melon cultivars including four Iranian local melon cultivars (Mashhadi, Shahde Shiraz, Khatooni and Khaghani) and two standard melon cultivars (Charentais *Fom1* and Charentais *Fom2*) were used in this study. Two specific primer pairs (PSh20-F/R and PSh20.2-F/R) were designed and used for the tracking of partial and full-length sequence of *Fom2*. More investigation was performed on the diversity occurrence pattern among cultivars and its conformity with cultivars resistive pattern against the fungus race 1. Our results showed that the partial and the full length sequence of *Fom2* had this potential to be tracked in sensitive as well as resistant cultivars. On the other hand, the cultivars reactions were not predictable based on the gene occurrence pattern. However, some studies have already been reported that the linked markers of *Fom2* were not useful for the prediction of reaction pattern in Iranian melon cultivars. The partial segments of *Fom2* amplified by PSh20-F/R primers were bi-directionally sequenced and the results were submitted in gene bank. Sequence analysis of NB-ARC domain in the six varieties led to detection of 16 polymorphic nucleotide positions, which nine of them appeared in protein levels. Six out of the nine mutations placed in AAA-ATPase subdomain. Although the polymorphic pattern of mutations in nucleotide and amino acid positions were not in accordance with the cultivars resistive pattern but the cultivars could be discriminated based on some other mutations. Due to the switching function of AAA-ATPase sub domain in ATP hydrolysis and activation of R proteins, variation in this region between the resistant and susceptible cultivars could be considerable. It seems the presence of identified mutations at AAA-ATPase subdomain of *Fom2* could be considered in cultivars resistive pattern unconformity and it needs to be followed in future studies to clarify the action of detected mutations in this subdomain.

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Published online: 22 March 2016

Keywords *Fom2* · NB-ARC domain · AAA-ATPase subdomain · Melon · Vascular wilt · *Fusarium oxysporum* f. sp. *melonis* · R gene diversity · Gene tracking · Functional mutations

Introduction

Iran after china and Turkey is the third producer of Melon (*Cucumis melo* L.) in the world (FAOSTAT 2013). Vascular wilt caused by *Fusarium oxysporum* f. sp. *melonis* (Leach & Currenu) Snyder & Hansen (FOM) is one of the most destructive soil-borne diseases in melon in Iran particularly in temperate and cold regions (Banihashemi 2010). Initial symptoms of *Fusarium* wilt often include a dull, gray green appearance of leaves that precedes a loss of turgor pressure and wilting. Yellowing of the leaves and necrosis will be occurred after wilting. The wilting generally starts with the older leaves and progresses to the younger foliage. Under conditions of sufficiently high inoculum density or a very susceptible host, the entire plant may wilt and die within a short time. Alive affected plants are often stunted and their yield have considerably been reduced. Under high inoculum pressure, seedlings may damp off as they emerge from the soil (Suarez-Estrella et al. 2004). Successively cropping of susceptible melon cultivars increase pathogenic populations and because of the persistent nature of this pathogen in soil the best way of disease control is by using *Fusarium*-resistant cultivars (Banihashemi and Dezeeuw 1975; Gordon et al. 1989; Wang et al. 2011). However, as resistant cultivars are utilized, new physiological races may develop in specific locations (Chikh-Rouhou et al. 2007). Four physiological races have been described for this pathogen and named: 0, 1, 2, and 1.2 (Mas et al. 1981). Two resistance genes, *Fom1* and *Fom2* have genetically been identified in melon against this pathogen. *Fom1* confers resistance to races 0 and 2 while *Fom2* confers resistance to races 0 and 1 (Banihashemi 2010). Race 1.2 is virulent to melon cultivars possessing resistance genes *Fom1* and *Fom2* and No genes have been identified in melons that confer high levels of resistance to race 1.2 (Risser et al. 1976) and other methods like grafting on resistant rootstocks have been proposed to limit the damage of the disease in some areas (Nisini et al. 2002). In Iran, Many efforts have been performed to identify resistance sources among Iranian cultivars in the last decades (Banihashemi and Dezeeuw 1975). Most Iranian cultivars are resistant to race 0 and all of them are susceptible to race 1.2 (Banihashemi 2010). Also enzymatic activity in roots of some Iranian local genotypes have been assayed to find their relation with resistance to the disease (Madadkhah et al. 2012). *Fom2* has been introduced as a key gene for conferring resistance to the race 1 in several studies (Oumouloud et al. 2009; Oumouloud et al. 2010; Risser et al. 1976; Zink and Thomas 1990). The gene linked markers to *Fom2* were identified and introduced in order to facilitate the next target which was the selection of resistant cultivars. (Wechter et al. 1995; Zink and Gubler 1985; Zink and Thomas 1990). The international standard cultivars M15 and M16 have been used as sensitive and resistant cultivars to the fungus race 1 in different studies (Oumouloud et al. 2009; Oumouloud et al. 2010). The full sequence of *Fom2* has been cloned and two following domains; NB-ARC (Nucleotide-Binding adaptor shared by Apaf1, certain R genes and CED4) and LRR (Leucine Rich Repeat) were observed in the gene protein sequence (Joobeur et al. 2004). The LRR domain has a direct role in pathogen effector proteins detection (Sela et al. 2012). The NB-ARC domain has three sub-domain which are altogether comprised a nucleotide binding site (Takken et al. 2006) and control the function of resistance gene via the ATPase role (Takken and Goverse 2012). The central NB-ARC domain in R proteins has been proposed to function as a molecular switch that, depending on the nucleotide bound, defines the activation state of the R protein (Rairdan and Moffett 2006; Takken et al. 2006; Tameling et al. 2006; Ade et al. 2007). In this functional model, the NB subdomain is the catalytic core, the ARC1 subdomain is required as a scaffold for the intramolecular interaction with the LRR, and the ARC2 subdomain is the regulatory element that transduces pathogen perception by the LRR into R-protein activation. So far, several mutations have been reported which are inactivated the function of this domain (Bendahmane et al. 2002; Grant et al. 1995; Van Ooijen et al. 2008). Performed analysis was suggested the presence of a sequence similar to LRR in NB-ARC domain (Wang et al. 2011). The effect of point mutations has been studied in the function of *I2* protein subdomains. This subdomain is responsible for resistance against *Fusarium oxysporum* f. sp. *lycopersici* race 2 (Tameling et al. 2006). The function and structure of *I2* gene is comparable with *Fom2* in many aspects.

LRR region in resistance genes has been investigated in many studies. Nucleotide and amino acid diversity have been shown at LRR region of *Fom2* (Joobeur et al. 2004) which could be affected on resistance to the fungus race 1 (Oumouloud et al. 2012; Wang et al. 2011). The occurrence of point mutations in resistance genes have also been reported in the other plant species (Mondragón-Palomino et al. 2002) and it is known as a mechanism to make the new type of resistance gene. Considering to the importance of NB-ARC domain in *Fom2* gene and the lack of functional information in this region, this domain was selected for sequencing, identifying possible mutations and to study their possible relationship of mutations with the type of reaction against the fungus races. The present study was carried out for the tracking of *Fom2* and to compare its diversity among local melon cultivars. Furthermore, the diversity occurrence pattern and its conformity with cultivars resistive pattern against *Fusarium oxysporum* f. sp. *melonis* race 1 were investigated.

Material and methods

Plant material

The seeds of some local and international melon cultivars were collected from different internal and external sources. The seeds were coded and stored in the seed bank of Plant Sciences Research Center, Ferdowsi University of Mashhad for the future use. They have shown different reaction to the physiological races of *Fusarium oxysporum* f. sp. *melonis*. The characterizations of cultivars used in this study have been presented in Table 1.

R: Resistant, S: sensitive, UK: Unknown.

Plant samples preparation and DNA extraction

The seeds of melon cultivars were disinfected by 70 % of ethanol for 40 s, washed twice by distilled water and germinated in humid tissue under fluorescent light at 25 °C in room temperature. Three germinated seeds from each cultivar were planted in a pot, moved to the green house with 16 h photoperiod and 60 % humidity at 22 °C temperature. The seedling true intact leaves were individually harvested, wrapped in Aluminum foil, flash froze in liquid nitrogen and stocked in −80 °C till DNA extraction. Genomic DNA was extracted from 1 g of leaf tissue using CTAB method (Cetyl Trimethyl Ammonium Bromide) with minor modification. The quality and quantity of extracted DNA were tested on 1 % agarose gel.

Biological data processing and primer design

The sequence of *Fom2* was retrieved from NCBI gene data bank. SMART software (http://smart.embl-heidelberg.de) was used to identify the available domains in the protein sequence of *Fom2*. Conserved regions in *Fom2* were detected by BlastX program (http://blast.ncbi.nlm.nih.gov) and specific area selected as primers match sites. Specific primers used for the partial and full-length tracking of *Fom2* were designed using Vector NTI V11 software, synthesized by MACROGEN, South Korea and listed in Table 2.

PCR protocol and gene tracking condition for partial and full length of *Fom2*

Reaction in total volume 10 μl was prepared by mixing about 50 ng genomic DNA, 0.5 μl of each primer (5 pmol), 1 μl 10X Tris-HCl buffer (GeNetBio, South Korea), 1 μl $MgCl_2$ (2.5 mM), 0.4 μl dNTP Mixture (200 μM each, Fermentas, Canada) and 0.24 μl Taq DNA polymerase (GeNetBio, South Korea, 1 U/μl). PCR reactions were performed in a thermocycler (Primus 25 Thermal Cycler, MWG Biotech Inc.) and the programs for two primer pairs used for the tracking of *Fom2* were presented in the Table 3. Amplification products were separated by gel electrophoresis on a 1 % agarose gel in 1X TAE buffer (Merck, Germany) at 85 V for 45 min, stained with DNA green viewer and visualized under UV light with an Gel analysis system (UviDoc, UK). The PCR products were purified using PCR Purification Kit (BioNEER, South Korea) following the procedure described by the manufacturer.

Sequence analysis

Partial *Fom2* amplicons were sequenced using related primers (Table 2) by MACROGEN, South Korea and sequencing results were analyzed using the following software: Chromas was used for the evaluation of sequencing quality, Seqman was utilized for assembling and consensus sequence identification and Mega5 used to detect diversity among different cultivars.

Results

Gene sequence analysis for *Fom2* and primer design

The gene coding sequence of *Fom2* was retrieved from NCBI (Accession number: DQ287965). It has been submitted according to the publication by Joobeur et al. 2004. The gene length is 3222 bp without any intron and codes a protein contained 1073 amino acid (ABB91438). Protein sequence analysis was performed using SMART software (http://smart.embl-heidelberg.de). The result showed that the domains NB-ARC and PLN03210 were located between amino acids 171–455 and 755–1020, respectively. In order to identify specific conserved motif region for primer design, the amino acid sequence of *Fom2* was checked by BlastX program (http://blast.ncbi.nlm.nih.gov). High homology has been observed between *Fom2* and five reported protein from *Cucumis melo* with following accession number: AAS80152.1, ABB91438.1, AAT77097.1, AAT77094.1 and AAT77098.1. The first two protein contained full sequence of *Fom2* and in the next three proteins, 541 beginner amino acids were missing where the sequence of NB-ARC domain was located at the same location (Fig. 1).

The similarity of the reported genomic sequences to the reported EST data from *Cucumis melo* has been investigated using BlastN program to make sure of the right selection for coding regions. The result showed a homology between EST-MU63031 and EST-MU66964. These two ESTs were connected to the following coding region of *Fom2*; 15–518 and 976–1578, respectively. Based on the aforementioned results the specific regions of *Fom2* were selected which were able to discriminate the gene's perfect form from its imperfect format. Furthermore, at the same region, the homologues areas with EST-MU63031 and EST-MU66964 were considered

Table 1 Characterization of local and international melon cultivars used in this study and their reported resistive pattern against physiological races of *Fusarium oxysporum* f. sp. *melonis*

Code	Name	Presented by	Subspecies	Race 0	1	2	1.2	Reference
M5	Mashhadi (Local)	Banihashemi, Z. University of Shiraz	*inodorus*	R	S	R	S	(Banihashemi 2010)
M6	Shahde Shiraz (Local)	Banihashemi, Z. University of Shiraz	*cantalupenses*	R	R	S	S	(Banihashemi 2010)
M8	Khatooni (Local)	Sobhani, A. R. Mashhad RCANR	*inodorus*	UK	UK	UK	UK	–
M9	Khaghani (Local)	Sobhani, A. R. Mashhad RCANR	*inodorus*	S	S	R	S	(Banihashemi 2010)
M15	Charentais *Fom1* (International)	Alvarez, J. M. CITA Spain	*cantalupenses*	R	S	R	S	(Oumouloud et al. 2009)
M16	Charentais *Fom2* (International)	Alvarez, J. M. CITA Spain	*cantalupenses*	R	R	S	S	(Oumouloud et al. 2009)

to select forward primer (PSh20-F) and reverse primer (PSh20-R), respectively (Fig. 1).

Tracking of the *Fom2* gene's sequence in Iranian local melon cultivars

The tracking of *Fom2* was carried out in the Iranian local mass melons using specific primers (PSh20-F/R). A band with 931 bp length was expected to be amplified using PSh20-FR primer pairs based on software analysis. One more primer pairs were also designed at the beginning and terminal part of gene coding sequence to amplify the full length of *Fom2*. Therefore, a 3222 bp fragment was expected to be amplified. The quality and quantity of extracted DNA from different cultivars were tested on electrophoresis gel and genomic DNA concentrations was estimated between 100 and 200 ng (Fig. 2a). The electrophoresis pattern of PCR product showed that *Fom2* was presented at all tested cultivars at the genome level (Fig. 2b). The presence of a single band in the range of 1 kbp was in agreement with the size of expected band (931 bp). As there are homologous resistance genes among different cultivars, the amplification of a single band shows the designed primers were so specific for this gene. PCR optimization was carried out by changing annealing temperature and performing touch down program. Higher amplification efficiency and thicker specific band for all cultivars except M5 were obtained after PCR optimization (Fig. 2).

Gene sequencing and nucleotide diversity identification in the partial sequences of *Fom2*

Bidirectional sequencing between two PSh20-F/R primer pairs for *Fom2* showed high similarity of the gene sequences among local melon cultivars. The results of multiple sequence alignment showed an overlap between 304 to 996 nucleotide positions in the obtained sequences. Moreover, 16 nucleotide diversities were observed in this area among different cultivars (Fig 3a). Constructed phylogenetic tree was able to discriminate samples in the two main groups (Fig 3b). The cultivars M5, M6, M8 and M16 were located in the first group. According to the cultivars resistive pattern, resistant and sensitive cultivars were located beside each other in the first group. In the second group, resistant and sensitive cultivars were also situated beside each other. This result showed that all mutations do not probably have a unique effect on the resistance or sensitivity occurrence. Therefore, the cultivars resistive patterns were studied and their mutation sites have been presented at the Table 4. In some positions such as 574, 620, 680 and 916 no difference was observed between standard resistant cultivars (Ref and M16) and sensitive cultivar (M15). Even in some positions, only one cultivar had a different sequence compared to others. For example, to the cytosine substitution by thymine could be referred in cultivar M16 at the positions 411 and 827. Special pattern was also observed for the cultivar M9 at the positions: 574, 620 and 916. Recurrence of these mutations in the cultivar population can be used for the cultivar discrimination. However, no correlation was observed between genes presence pattern and the type of cultivars reaction against *F. oxysporum* f. sp. *melonis* (race1). Comparison of different mutation sites in the codon level showed that the third base of codon has been mutated at nine positions (Table 4). Since all mutations are not appeared at the protein level, the diversity of amplified fragment for different cultivars has to be studied at the protein level.

Table 2 Sequence of the primers designed for the partial and full-length tracking of *Fom2*, their annealing temperatures (T_m) and targets

Primers	Sequences (5′- 3′)	T_m (°C)	Target (bp)
PSh20-F	GGATGTGGGTGGATGATCTTCGG	67.1	931
PSh20-R	CGTGCAACCAATGGTACACCACC	66.8	
PSh20.2-F	TCAAAAAAACCTACAACTCTCGAACG	63.6	3222
PSh20.2-R	ATGGGTGATTTCCTATGGACTTTTG	58.6	

Table 3 PCR programs for two primer pairs used for the tracking of *Fom2*

Pre-denaturation		93	180
Steps for PSh20-F/R		Temperature (°C)	Time (Sec)
35 cycles	Denaturation	92	45
	Annealing	58	30
	Extension	72	60
Termination		72	300
Steps for PSh20.2-F/R		Temperature (°C)	Time (Sec)
3 cycles	Denaturation	92	45
	Annealing	66	30
	Extension	72	60
3 cycles	Denaturation	92	45
	Annealing	65	30
	Extension	72	60
3 cycles	Denaturation	92	45
	Annealing	64	30
	Extension	72	60
3 cycles	Denaturation	92	45
	Annealing	63	30
	Extension	72	60
3 cycles	Denaturation	92	45
	Annealing	62	30
	Extension	72	60
Termination		72	300

Amino acid diversity identification in the *Fom2* partial protein sequences

Multiple sequence alignment for protein sequences showed that diversity in nucleotide sequences were only appeared at nine positions at protein level (Fig. 4a), so that at six positions only one sequence showed difference with other sequences. The cultivar M9 showed different sequence at the positions; 192, 207 and 306. At the position 227 in the cultivar M6 unlike to the other cultivars, Valine was substituted by Alanine. Our results showed that, all the seven observed nucleotide diversities in *Fom2* have not been made any changes at coding amino acid sequences of this gene. Constructed protein base tree has been clustered cultivars completely similar to nucleotide base tree (Fig. 4b). No specific pattern was identified between resistant and sensitive cultivars from the amino acid type point of view (Table 5). There were four diverse amino acid positions out of nine different identified positions in the resistant cultivars. While identical amino acid were only observed at three positions in the sensitive cultivars.

The effect of each mutation on resistance gene function could be investigated by identification of the mutation site in functional domain. The reports have been shown that mutation occurrence in various domains and sub-domains would have different effects (Tameling et al. 2006; Van Ooijen et al. 2008). In order to find the sites of observed mutations in the melon cultivars, the sub-domains position related to NB-ARC domain in the protein sequence of *Fom2* was identified by multiple alignment with the gene *I2* protein (Van Ooijen et al. 2008) (Fig. 4a). The investigation on the mutation sites was showed; eight mutations out of the nine occurred mutations have been located in NB-ARC domain at protein level. Meanwhile, six mutations have been occurred in AAA-ATPase sub-domain and the ARC1 region was identical among all samples.

Discussion

In the present study, the gene *Fom2* was tracked in the local melon cultivars. Moreover, nucleotide and amino acid diversity in partial sequences of this gene were compared between resistant and sensitive melon cultivars. Specific primers were designed based on the *Fom2* reported sequence with following accession number: DQ287965 (Joobeur et al. 2004) and used for the gene tracking and identifying gene presence pattern in different cultivars. The gene linked markers to *Fom2* were also identified and they have been introduced in order to facilitate the next target which was the selection of resistant cultivars against the race 1 of *Fusarium oxysporum* f. sp. *melonis* (Wechter et al. 1995; Zink and Gubler 1985; Zink and Thomas 1990). It has to be stated that the linked markers of *Fom2* have already been used for the selection of resistant genotype in the different studies (Wang et al. 2000; Wechter et al. 1998; Wechter et al. 1995; Zheng et al. 1999; Zink and Gubler 1985; Zink and Thomas 1990).

The results of the present study showed that the full sequence of *Fom2* was traceable in our local resistant and sensitive cultivars at the genome level. In different studies the

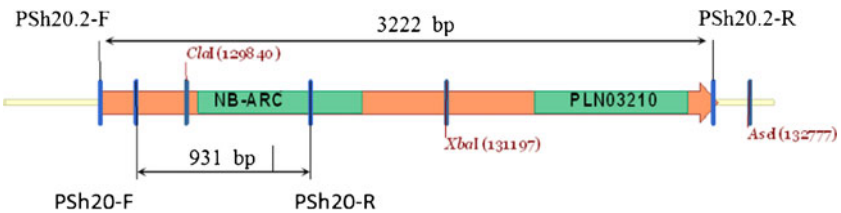

Fig. 1 Schematic presentation of the gene *Fom2* in the melon genome. The domains NB-ARC and PLN03210, the position of the PSh20-F/R and PSh20.2-F/R primer pairs and the length of Full and partial gene amplicons have been shown (Vector NTI V.11)

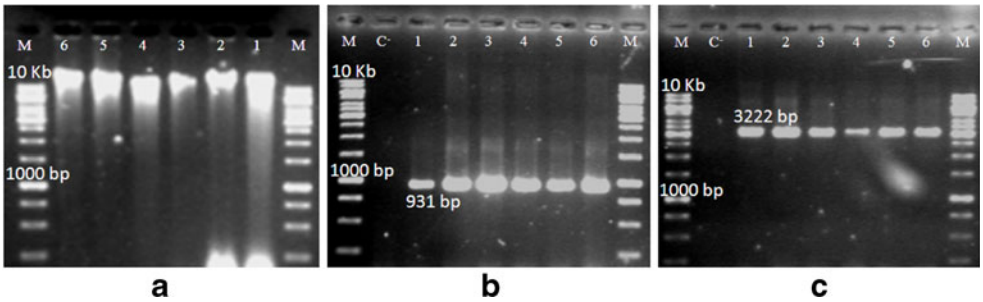

Fig. 2 Electrophoresis results for extracted genomic DNA, 2 μl from each samples were loaded in the wells (**a**). Gene tracking for the partial sequence of *Fom2* using PSh20-F/R primer pairs which was given a single band with 931 bp length as target, 2 μl from the obtained PCR products was loaded in the wells (**b**). Gene tracking for the full sequence of *Fom2* identified in resistant and susceptible melon cultivar to *Fusarium oxysporum* f. s*p. melonis* (race1). PCR was performed using PSh20.2-F/R primer pairs which was amplified a single band for the full sequence of *Fom2* with 3222 bp length (**c**). 1, 2, 3, 4, 5 and 6 are melon cultivars with the following codes; M5, M6, M8, M9, M15 and M16, respectively

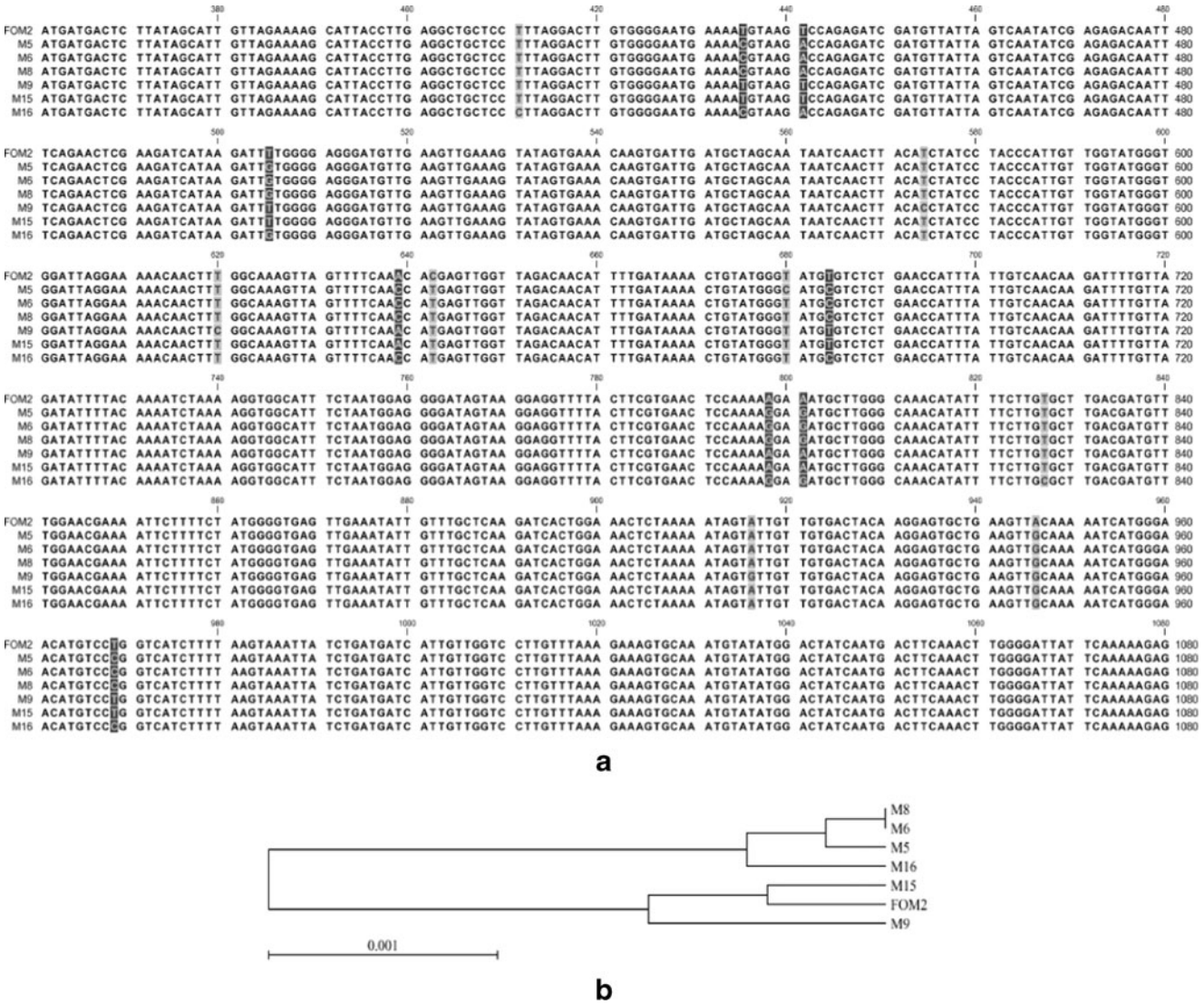

Fig. 3 Multiple sequence alignment for the *Fom2* partial nucleotide sequences tracked from local and international melon cultivars (**a**). UPGMA constructed phylogenetic tree is showing the affinity among cultivars (**b**). The sequence of *Fom2* (Accession number: DQ287965) from a resistant plant (PI161375) was aligned as a reference. M5, M6, M8, M9, M15 and M16 are the tracked sequences from local and international melon cultivars. The numbers show the nucleotide positions to the gene's start codon. The gray and black backgrounds illustrate nucleotide replacement for different cultivars

Table 4 Nucleotide position and nucleotide diversity in the partial sequences of *Fom2* from different melon cultivars

			Nucleotide position																
Row	code	R1	411*	435*	441*	505	574	620	639*	642*	680	684*	798*	801*	827	916	946	969*
1	Ref	R	T	T	T	T	T	T	A	C	T	T	A	A	T	A	A	T
2	M6	R	T	C	A	G	T	T	C	T	T	C	G	G	T	A	G	C
3	M16	R	C	C	A	G	T	T	C	T	T	C	G	G	C	A	G	C
4	M5	S	T	C	A	G	T	T	C	T	C	C	G	G	T	A	G	C
5	M9	S	T	T	T	T	C	C	A	T	T	T	A	A	T	G	G	T
6	M15	S	T	T	T	T	T	T	A	T	T	T	A	A	T	A	G	T
7	M8	UK	T	C	A	G	T	T	C	T	T	C	G	G	T	A	G	C

Ref: The sequence of *Fom2* (Accession number: DQ287965) from a resistant plant (PI161375) used as a reference sequence

R Resistant; *S* Sensitive; *UK* Unknown; *: Codon third position; *A*, *C*, *G* and *T* Adenine, Cytosine, Guanine and Thymine

international standard cultivars, M15 and M16 have been used as sensitive and resistant cultivars to the fungus race 1, respectively (Oumouloud et al. 2009; Oumouloud et al. 2010). Therefore it was expected, these two cultivars must be different from the *Fom2*'s presence aspect. Our investigation showed that the sequence of *Fom2* was traceable in both cultivars. Therefore, resistant and sensitive Iranian local melon cultivars could not be discriminated based on the gene presence pattern. It has also been reported that, the presence pattern of *Fom2*'s linked markers and resistive pattern of this gene have not shown conformity in the following cultivars; Sisi, Persia 202 and Meshad (Dean and Wang 2001). Another study showed that the partial sequence of *Fom2* in three sensitive cultivars named, Ve'drantais, AY and Durango was able to be amplified like a resistant line called PI161375 (Joobeur et al. 2004). The results have been proposed this hypothesis; the difference between resistant and sensitive cultivars is hidden inside the sequence of *Fom2* at nucleotide and protein level.

In the present study, the partial domain of NB-ARC was used in order to design specific primers (PSh20-F/R) to study the diversity between resistant and sensitive cultivars. Although, there are two domains; NB-ARC and LRR in the protein sequence of *Fom2* (Joobeur et al. 2004) but concerning to the direct role of LRR domain on the interaction with pathogen effector proteins (Sela et al. 2012), more attention has been paid to this region of domain so far. Consequently, several resistance effective mutations have been identified at LRR region (Oumouloud et al. 2012; Wang et al. 2011). The occurrence of point mutations in the resistance genes has been reported in the other plant species as well (Mondragón-Palomino et al. 2002) and this was known as a mechanism to make the new type of resistance gene.

In this paper, the diversity between resistant and sensitive cultivars has been investigated for the partial sequence of *Fom2* at nucleotide and protein level. Sequencing of this region was leaded to the identification of 16 mutated sites among cultivars (Fig. 3a). Although, special pattern was obtained for a number of mutations to discriminate some cultivars from the others (Table 4) but the pattern of mutations were not unique in the studied resistant and sensitive cultivars. Based on the constructed phylogenetic tree no conformity was shown between cultivars affinity and their resistive pattern (Fig. 3b). Furthermore, No direct correlation was observed between mutations occurrence pattern and the reaction type of each cultivars against fungus race1. This unconformity has already been reported for the identified point mutations at LRR region in resistant and sensitive cultivars (Oumouloud et al. 2012). The incidence of some mutations on the third base of codons reduced the probability of mutation occurrence at protein level. The mutations which were inactivated the function of NB-ARC domain have been reported in several studies (Bendahmane et al. 2002; Grant et al. 1995; Van Ooijen et al. 2008). Our studies on the protein sequence diversity was shown that the incidence pattern of mutations in amino acid sequence related to investigated fragment could also not created a distinct pattern for resistant and sensitive cultivars. Another study was performed on LRR region of *Fom2* and

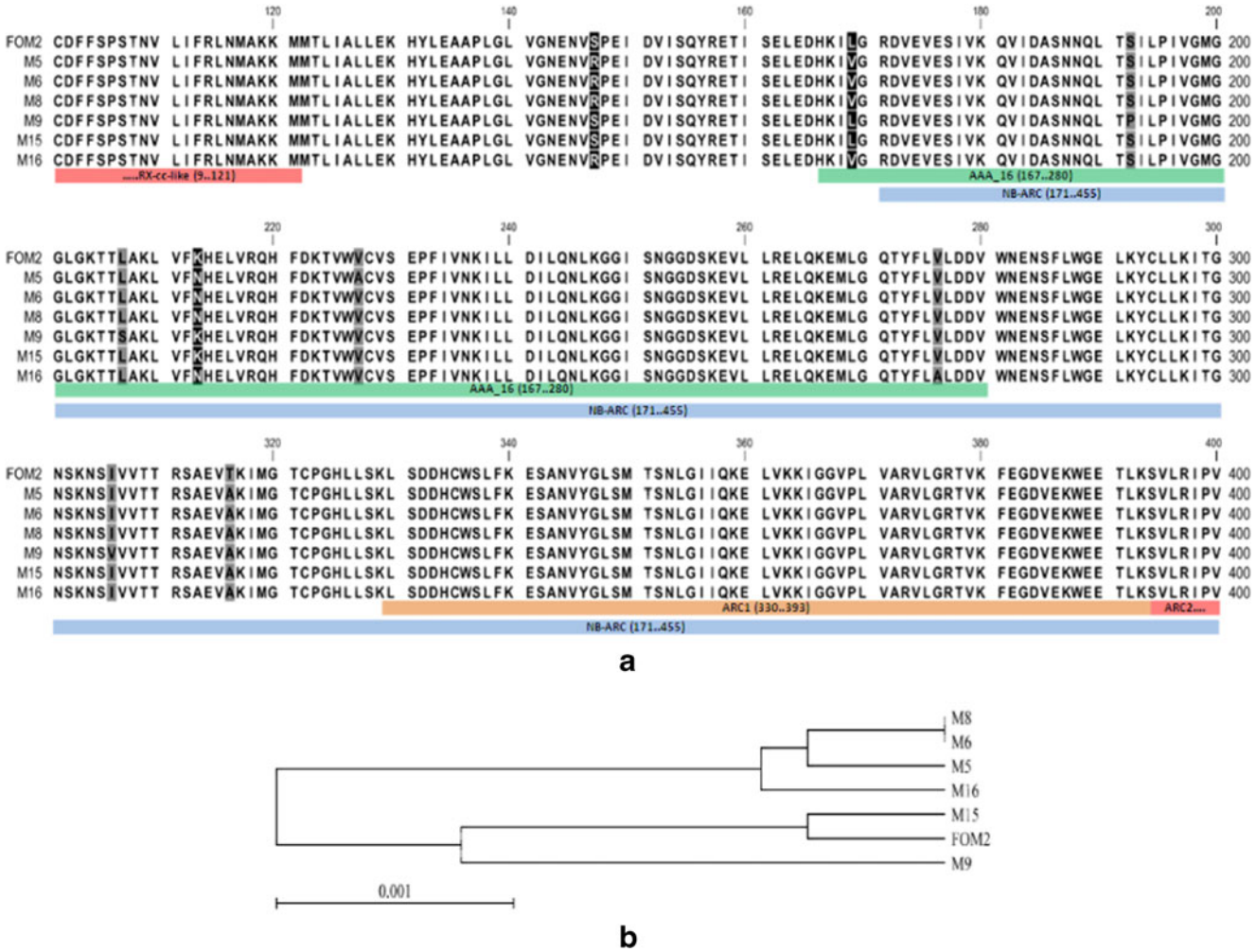

Fig. 4 Multiple sequence alignment for the *Fom2* partial amino acid sequences from the local and international melon cultivars (**a**). UPGMA and protein base constructed phylogenetic tree, which is showing the affinity among cultivars (**b**). The amino acid sequence of *Fom2* reported from a resistant plant (PI161375) used as a reference. M5, M6, M8, M9, M15 and M16 are the protein sequences from local and international melon cultivars. The numbers show the position of amino acids to the gene start codon. The gray and black backgrounds illustrate the amino acid replacement for different cultivars. Red cadre is terminal part of Coiled-coil domain. Blue cadre is part of NB-ARC domain including AAA-ATPase sub-domain (green), ARC1 and ARC2

showed that, there was this possibility to identify the functional point mutations which are important in the function of resistance gene (Wang et al. 2011). In the present study, the diversity between a resistant American melon and a sensitive Chinese melon was studied. Furthermore, the manner of segregation has also been studied among their offspring in F2 population.

According to this, the discriminator positions could be observed in the cultivars; M15 and M16 which are sensitive and resistant to the race 1, respectively. As it has been presented in the Table 5, in M16 (resistant cultivar) the following amino acids Arg, Val and Asn, while in M15 (sensitive cultivar) the following amino acids Ser, Leu and Lys could be observed at the positions; 147, 169 and 213, respectively. In the other study the sequence of LRR region from the gene *Fom2* was compared in resistant and sensitive cultivars against the fungus race 1. Significant mutations have been introduced which were able to discriminate a number of resistant cultivars from a sensitive cultivar (Oumouloud et al. 2012). In our presented results at the Table 5 there are this possibilty to discriminate two resistant cultivars (M6 and M16) from M15. The interesting point according to the Table 1 is that, these three cultivars are belonging to the subspecies of *cantalupenses* and M6 is a local resistant cultivar. As it has already been referred the performed studies on the *Fom2*'s protein was only restricted on LRR domain while in the performed studies on the *I2*'s gene have been paid more attention to the effect of mutations in NB-ARC domain (Tameling et al. 2006). NB-ARC domain could be interested because of the role of ATPase in resistance gene activation (Takken and Goverse 2012). Considering to the functional and structural similarities between *I2* and *Fom2* the possibility of mutations effect were noticed in their

Table 5 Positions and diversity of amino acids in the partial coding sequences of Fom2 protein from different melon cultivars

			Amino acid position								
Row	Code	R1	**147**	**169**	**192**	**207**	**213**	**227**	**276**	**306**	**316**
1	Ref	R	Ser	Leu	Ser	Leu	Lys	Val	Val	Ile	Thr
2	M6	R	Arg	Val	Ser	Leu	Asn	Ala	Val	Ile	Ala
3	M16	R	Arg	Val	Ser	Leu	Asn	Val	Val	Ile	Ala
4	M5	S	Arg	Val	Ser	Leu	Asn	Val	Val	Ile	Ala
5	M9	S	Ser	Leu	Pro	Ser	Lys	Val	Val	Val	Ala
6	M15	S	Ser	Leu	Ser	Leu	Lys	Val	Val	Ile	Ala
7	M8	UK	Arg	Val	Ser	Leu	Asn	Val	Ala	Ile	Ala

Ref: The protein sequence of Fom2 used as a reference sequence

R Resistant; *S* Sensitive; *UK* Unknown

domains and sub-domains. The comparison results showed that the selected region from the protein of *Fom2* is included the terminal part of coiled-coil domain and a part of NB-ARC domain including the following sub-domain; AAA-ATPase, ARC1 and ARC2. It is remarkable that eight out of the nine occurred mutations are located in NB-ARC domain at protein level. Herein six mutations were taken place at AAA-ATPase sub-domain. Several studies have been shown the activity of AAA-ATPase domain is essential for the emergence of plant defense response (Qi and Innes 2013). It has also been demonstrated some of mutations in this sub-domain could be leaded to resistance gene autoacivation (Williams et al. 2011). Therefore, the incidence of these mutations in the number of cultivars have probably suggested a complicated role for the resistance occurrence pattern. Based on the obtained results from the available models (Rairdan and Moffett 2006; Tameling et al. 2006) it can be concluded; in the number of cultivars provided that the presence of resistance gene several process would be essential for the emergence of resistance. The first object which should be provided is the possibility to detect pathogen effector proteins using LRR (Leucine Rich Repeats) sequences. The mutation occurrence at LRR domain could be important in this step. It has been shown this step and its occurred mutations at LRR domain have to be considered for resistant and sensitive cultivars discrimination (Oumouloud et al. 2012; Wang et al. 2011). From the other side, NB-ARC domain must be able to make a connection with ATP and actively change R protein 3D-structure so that desired condition turn out to be provided for pathogen effector proteins detection and plant defense system induction (Qi and Innes 2013). It seems the presence of identified mutations at AAA-ATPase sub-domain of the gene *Fom2* could be considered in cultivars resistive pattern unconformity. However, confirmation of these effects needs to be more investigated.

Acknowledgment This work was financially supported by the Deputy of Research of Ferdowsi University of Mashhad, which is gratefully acknowledged. We thank from the Plant Research Centre Management for providing experimental facilities for this project.

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