Study of new NBS-LRR genes analogues in cucurbits native types in Iran

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Abstract

Nucleotide binding site leucine-rich repeats (NBS-LRR) accounting for the main disease resistance proteins play an important role in plant defense against pathogen attack. The current study aimed to identify new NBS-LRR gene members in native types of cucurbit species in Iran. Accordingly, DNAs of melon, cucumber and cantaloupe native types to Iran were identified using three primer pairs. PCR products of the expected size were generated and the obtained DNA was cloned using the pGEM-T. BLASTN algorithms were used to compare the insert sequences with sequences available at the Entrez nucleotide and protein. Phylogenetic analyses were conducted using the MEGA software. The results analysis suggests that cucurbit species native types' genome contain new NBS_LRR resistance gene analogues against cucurbit pathogens. These resistance gene analogues are composed of genes related to both coiled-coil (CC) and toll-interleukin-receptor homology (TIR) domain containing NBS-LRR R-genes. Phylogenetic analysis of these genes shows that they include NBS-LRRs derived from a recent common ancestor. This study provides an insight into the evolution of NBS-LRR genes in the Cucurbit native type species genomes that are resistance analogues against cucurbit bacterial blight pathogen and tomato mosaic virus.

Key words: Cucurbits species, Iran, Native types, NBS-LRR.

INTRODUCTION

Cucurbitaceae family includes Cucumbers (*Cucumis sativus* L.), squash (*Cucurbita moschata* Duch.), melon (*Cucumis melo* L.), bottle gourd [*Lagenaria siceraria* (Mol.) Standl.], and watermelon [*Citrullus lanatus* (Thunb) Mansfeld] (Helm and Hemleben, 1997; Sanjur *et al.*, 2002; Levi *et al.*, 2005; Sikdar *et al.*, 2010). Until recently, it was thought that *Cucumis* genus had 32 species and was probably domesticated in Africa (Ghebretinsae and Barber, 2006; Chen *et al.*, 2010). However, broader studies revealed that *C. sativus* is closer to 13 species from Australia, India, Yunnan and Indochina than to any African species (Renner and Schaefer, 2008). Biogeographic inference for economically important plants has become complicated by human transport of seeds between continents for at least 10000 years. In the economically important plant family Cucurbitaceae, these difficulties have led to the broad geographical distribution of their close relatives, watermelon, cucumber, loofah, bitter gourd, etc. (Smith, 1997; Dillehay *et al.,* 2007). Over the recent years, research shows that Cucurbitaceae initially diversified in Asia (specifically, the region north of the Tethys) sometime in the Late Cretaceous (Chakravarty, 1946) followed by the repeated spread of lineages into the African, American and Australian continents via transoceanic long-distance dispersal (LDD) (Chakravarty, 1959).

Although Cucurbitaceae plant family is the most important vegetable crops in the world, their susceptibility to multiple pathogens inhibits their

Table 1. PCR amplification result and Blast search.

Identified RGA used as a query	Species	Primer combination	Band size	Class amplified
TN-94-139	*Cucumis sativus*	F1/R1	450	TIR-NBS-LRR
TN-94-133	*Cucumis sativus*	F1/R1	450	non TIR
TN-94-148	*Cucumis sativus*	F1/R2	400	TIR-NBS-LRR
TN-94-197	*Cucumis sativus*	F1/R1	450	TIR-NBS-LRR
TN-94-163	*Cucumis sativus*	F1/R1	450	non TIR
TN-92-827	*Cucumis melo*	F1/R1	450	non TIR
TN-92-325	*Cucumis melo*	F2/R2	350	non TIR
TN-92-311	*Cucumis melo*	F2/R2	350	non TIR
TN-92-342	*Cucumis melo*	F2/R2	350	non TIR
TN-92-324	*Cucumis melo*	F2/R2	350	non TIR
TN-92-107	*Cucumis melo* var . Cantalupensis	F2/R2	350	non TIR
TN-92-99	*Cucumis melo* var . Cantalupensis	F2/R2	350	TIR-NBS-LRR
TN-92-115	*Cucumis melo* var . Cantalupensis	F2/R2	350	non TIR
TN-92-80	*Cucumis melo* var . Cantalupensis	F2/R2	350	TIR-NBS-LRR
TN-92-120	*Cucumis melo* var . Cantalupensis	F2/R2	350	non TIR

production increase and quality improvement (Palmer and Williams, 1981; Wyszogrodzka *et al*., 1987; Schaefer *et al*., 2009). The best way for fighting against different pathogens in different plants is using resistance sources. The resistance mechanism in plants is activated by resistance genes. Nucleotide-binding site (NBS)-leucine-rich repeat (LRR) proteins in plants encoded by resistance genes play an important role in plants response to various pathogens, including viruses, bacteria, fungi, and nematodes (Ellis and Jones, 1998). Some resistance gene analogues in cucumbers including NBS and Pto had been previously isolated and characterized using degenerate primers (Wan and Chen, 2010; Wan *et al.,* 2010). Recently, the cucumber scab R gene *Ccu* has been localized into an R-gene cluster located in a 670 kb region of cucumber chromosome 2 (Kang *et al*., 2011).

In another study, four resistance gene homologues (RGHs) were located in the region delimited by the molecular markers Indel 01 and Indel 02, and thus were possible *Ccu* candidates (Kang *et al*., 2011). In the present study, a complete set of NBS-LRR proteins were identified from the whole genome data set of Cucumbers (Wan *et al.,* 2013). However, little resistance associated with genetic and genomic resources is available for the improvement of these crops and this experimental approach failed to detect the members of these gene families in the cucurbit native types especially in Iran.

There are a wide genetic distance among cucurbit species such as the *Citrullus* and *Cucumis* (Sikdar *et al.*, 2010). Moreover, it was reported that *Cucumis* and *Cucurbita* are more closely related to each other than any of them to *Luffa* (Schaefer *et al*., 2009). Therefore, the analysis of R-genes or RGHs will contribute to their timely application in disease resistance breeding in Cucurbitaceae crops (Dracatos *et al.,* 2009). The current study aimed to isolate, identify and analyze resistance genes from Cucurbit species native types in Iran using molecular techniques.

MATERIALS AND METHODS

Plant materials and DNA isolation

Seeds for the Iranian Cucurbit native types (TN-94-163, TN-94-197, TN-94-148, TN-94-133, TN-94-139, TN-92-324, TN-92-342, TN-92-311, TN-92-325, TN-92-827, TN-92-120, TN-92-80, TN-92-115, TN-92-99, TN-92-107) related to three species (Table 1) were prepared from Iranian biological resource center (IBRS). Seeds were grown in growth chambers at 25 °C for 12 h (day) and 18 °C for 12 h (night). Relative humidity was maintained at 65– 75%. Young leaves were harvested from 4 week-old plants, immediately frozen in liquid nitrogen and then stored at −80 °C for nucleic acid extraction. Total genomic DNA was isolated using the modified CTAB protocol as described by Doyle and Doyle, (1990).

Primers and PCR conditions

NBS encoding RGHs from melon, cucumber and cantaloupe native types to Iran were also identified via degenerate PCR amplification using 3 pairs of degenerate primers. The primers were designed by the previous researchers based on the conserved regions of P-loop and GLPL of amino acid identity among the known NBS-LRR R genes from the other plant species,

Table 2. Degenerate primers used for PCR amplification from four major *Cucurbitaceae* crops.

Primer	Conserved motif	Primer sequence	Reference
F1	P-loop	TGSSRGGHWYRGGBAAAACTAC	Zhang *et al.* (2008)
R1	GLPL	HRCWARAGGVARCCCTYBACA	
F2	P-loop	GGDGTDGGNAARACWAC	Deng *et al.* (2000)
R2	GLPL	IARIGCIARIGGIARNCC	
F3	P-loop	GGWATGGGWGGWRTHGGWAARACHAC	Lee *et al.* (2003)
R3	GLPL	ARNWYYTTVARDGCVARWGGVARWCC	

The sequences are coded according to the International Units of Biochemistry: N = A or C or G or T; R = A or G; S = C or G; Y = C or T; W= A/T; Y= T/C; B= G/C/T; H=A/T/C; V = A or C or G; I = Inosine.

as listed in Table 2.

PCR reactions were carried out in a total volume of 25 µl containing 20 ng template DNA, 0.25 µM of each primer, 1.5 mM $MgCl_2$ and 0.5 U of *Tag polymerase* (Sinagen, Iran). Cycling conditions consisted of a 3 min initial denaturation at 94 °C followed by 40 amplification cycles consisting of 94 °C for 1 min, 45 to 60 °C (Table 1) for 45 s and 72 °C for 1 min and a final extension of 72 °C for 7 min. PCR products were separated by electrophoresis on 1% agarose gel and stained with ethidium bromide for visualization.

Cloning and analysis of PCR products

Bands of the expected size of amplification were excised from the gel and purified using the PCR elution kit (Fermentas, Netherland). The obtained DNA was cloned using the pGEM-T Easy vector system following the manufacturer's instructions and transformed into *Escherichia coli* DH5α. Recombinant plasmid DNA was extracted using alkaline lysis, and digested with *Eco*RI to verify the presence of the expected insert. Plasmid DNA was purified with a plasmid extraction kit (Vivantis, Malaysia).

Sequencing and phylogenetic analysis

The obtained sequences were first exposed to the VecScreen algorithm (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) in order to remove the contaminating vector sequences. BLASTN algorithms (Altschul *et al.,* 1990) were used to compare the insert sequences with sequences available in the Entrez nucleotide and protein databases (http://www.ncbi.nih.gov/BLAST). The CLUSTALW algorithm (Thompson *et al*., 1997) was used for the multiple sequence alignments and the BL2SEQ algorithm (Altschul *et al.,* 1990) was used for comparing two amino acid sequences to each other. Phylogenetic analyses were conducted using the MEGA software (version 6) (Tamura *et al*., 2011). Robustness of clustering was checked by bootstrapping 100 replicates. Searches for ORF were done using ORF finder at the NCBI server. Branch lengths were assigned by pairwise calculations of the genetic distances, and missing data were treated by pair wise deletions of the gaps.

Bioinformatic analysis of proteins related to 325 and 197

In order to study protein sequences, candidate resistance gene analogues 325 and 197 were choosen for bioinformatic analysis because they are potentially resistance analogues against *Pseudomonas syringae,* one of most important cucurbit pathogens in the world (Wan and Chen, 2010). These protein sequences were analyzed using the Universal Protein Resource KnowledgeBase (UniProtKB) (http://www.uniprot.org/) and the intracellular, extracellular and membrane portions of candidate sequences were performed by TMHMM program (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The first and second structure of the proteins 325 and 197 were determined using Uniprot B, PSIpred programs, and their location was purposed by PSORT PROTEIN.

RESULTS

Amplification, identification, and characterization of NBS fragments

PCR products of the expected size (~400 bp) were generated by each of the three primer sets with Iranian cucumber native types. Primer sets F2/R2 generated a major band of around 350 bp and a few faint bands (Figure 1). Primer sets F1/R1 produced a major band of 450 bp and a few faint bands in some of Iranian cucumber native types. Therefore, PCR products of 350 bp in length generated by the primer sets F2/R2 were cloned separately from each Iranian cucumber native types. The primer set F2/R2 gave the best results, as it could amplify 5 out of the total 10 NBS encoding sequences from all of the tested native types.

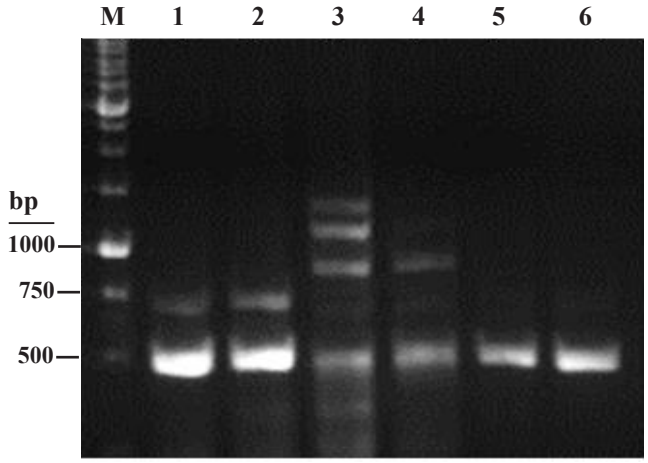

Figure 1. PCR amplification products generated by the selected resistance gene degenerate primer (F2/R2) in six major *Cucurbitaceae* crops. *Lanes 1- 6* amplicons generated by primer combinations of F2 and R2; lane M related to marker 1Kb (Fermantase, Leon-Rot, Germany).

Five recombinant (white) clones from each ligation reaction were randomly chosen to be further characterized by sequencing. BLAST search revealed that only five sequences were encoded open reading frames while the remaining sequences did not exhibit any significant similarities to any of the publicly available proteins. Homology searches of the GenBank database revealed that the obtained NBS sequences exhibited significant similarities with previously identified NBS: AEV46188, AEV46164 and XP-008463577 from *Cucumis melo*, ACX80257 from Cucumis x hytivus and from *Citrullus lanatus*.

The remaining unique NBS sequences were identified as resistance gene candidates by the presence of an uninterrupted open reading frame and by characteristic R-gene NBS motifs (Pan *et al*., 2000). Further, conceptual translations were used to query the NCBI non-redundant, conserved domain, and the Arabidopsis Genome Initiative protein databases.

Phylogenetic analysis of cucumber NBS-encoding genes

The NBS region, however, is highly conserved and is often used to generate multiple sequence alignments and phylogenetic tree constructions (Nair and Thomas, 2012). In order to elucidate the relationships among NBS-encoding genes from Iranian cucumber native types, we selected 15 cucurbit resistance gene analogues (RGAs) identified in this study, as well as reference R genes belonging to subclasses TIR and non-TIR R proteins to construct a neighbor-joining (NJ) phylogenetic tree. These genes were divided into two families, the CC-NBS and TIRNBS, which are supported by the high bootstrap values (Figure 2). This was consistent with the results reported by Pan *et al.* (2000), which showed that both the TIRNBS and CCNBS families of genes occur in dicotyledonous plant species. In addition, it was observed that the members in CCNBS were more numerous than those in the TIR-NBS family, which are composed of 10 and 5 members, respectively. Recently, melon genome sequence was available (Garcia-Mas *et al*., 2012) and NBS-LRR family of cucumber was studied. The same distribution of members of TIR-NBS and NBS-LRR family members was also found in melon (Pan *et al*., 2000).

Comparative analysis of NBS-encoding genes from Iranian cucurbit native types and Cucurbitaceae crops

In this study, both the TIR- and CC-NBS families were identified in all genes from the Iranian cucumber native types (Figure 2). To gain an insight into the phylogenetic relationship of NBS-encoding genes in Iranian cucumber native types, database mining and PCR amplification were employed. Phylogenetic analysis of NBS-encoding genes and RGHs in Iranian cucumber native types and RGHs in the Cucurbitaceae crops (Figure 2) shows that NBS-LRR sequences isolated from TN-94-148, TN-92-32,4 TN-92-313, TN-92-325 and TN-94-197 are related to NBS-LRR resistance protein (AEV46181) from *Citrullus lanatus* and act as a resistance gene against *Pseudomonas syringae*. NBS-LRR sequences isolated from TN-92-99 are related to NBS-LRR resistance protein (XP_008463577) from *Cucumis melo* and are TMV N-like resistance protein.

Bioinformatic analysis of proteins related to 325 and 197

Investigation of the second structure of the analog proteins 325 and 197 from Iranian native type cultivars, TN-92-325 and TN-92-197, using PSIpred showed that these proteins consists only of α helices (Figure 3). Bioinformatic analysis related to protein targeting, using PSORT PROTEINT software showed that the highest level of protein incorporation was in the plasma membrane and its extracellular secretion was low. Both of them share similarity with an NBS-LRR protein in *Citrullus lanatus* and are potentially involved in response to *Pseudomonas syringae.*

DISCUSSION

Numerous plant disease resistance genes including NBS-LRR genes that play a major role in resistance against diverse range of pathogens have been identified

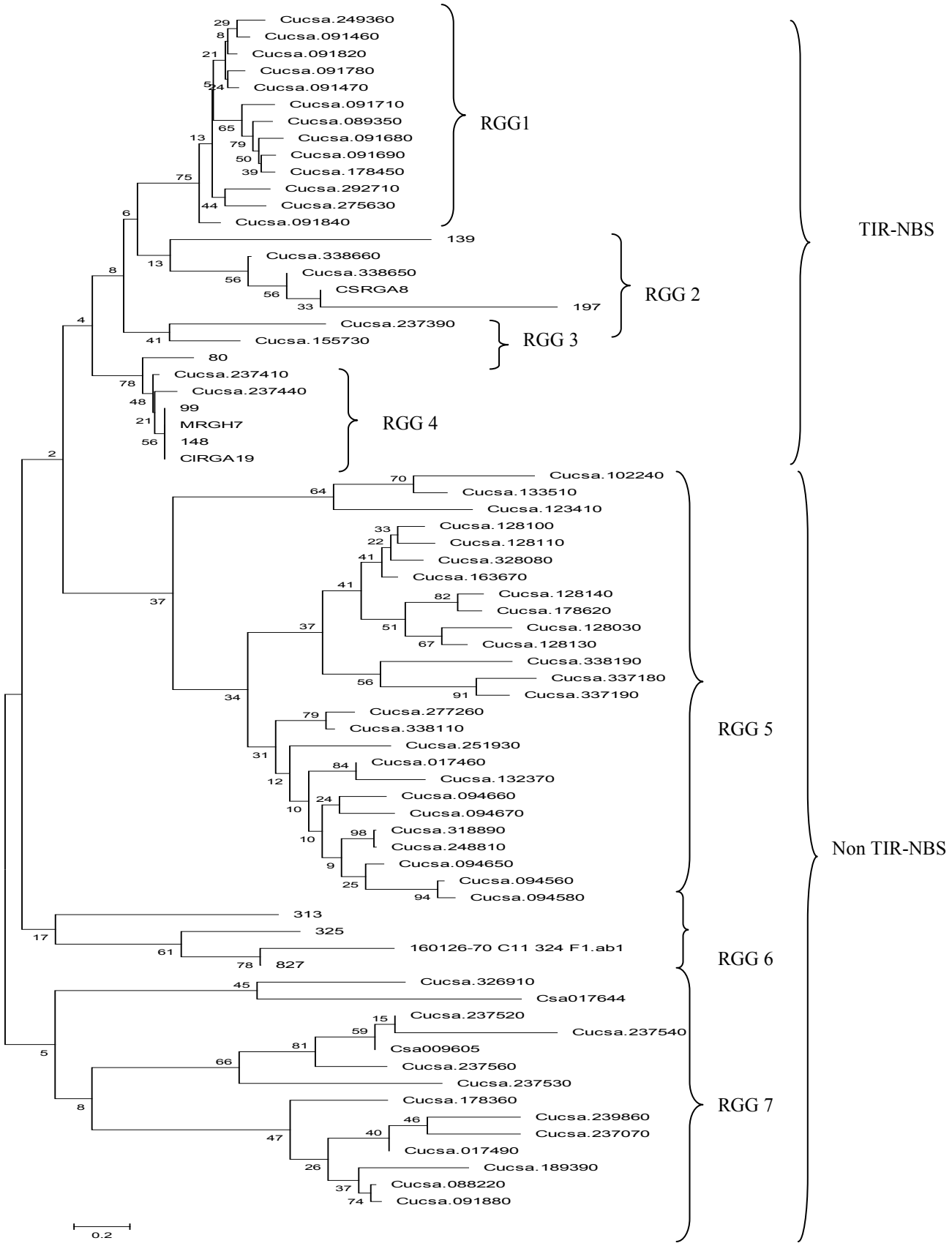

Figure 2. Phylogenetic analysis of NBS-encoding genes in cucumbers using MEGA 6.0. Phylogenetic tree was constructed using the NBS domain sequences of this study and 63 NBS sequences from NCBI gene bank. The scale represents the average number of substitutions per site. The numbers on the branches indicate the percentage of 1000 bootstrap replicates that support the node with only values >50% reported.

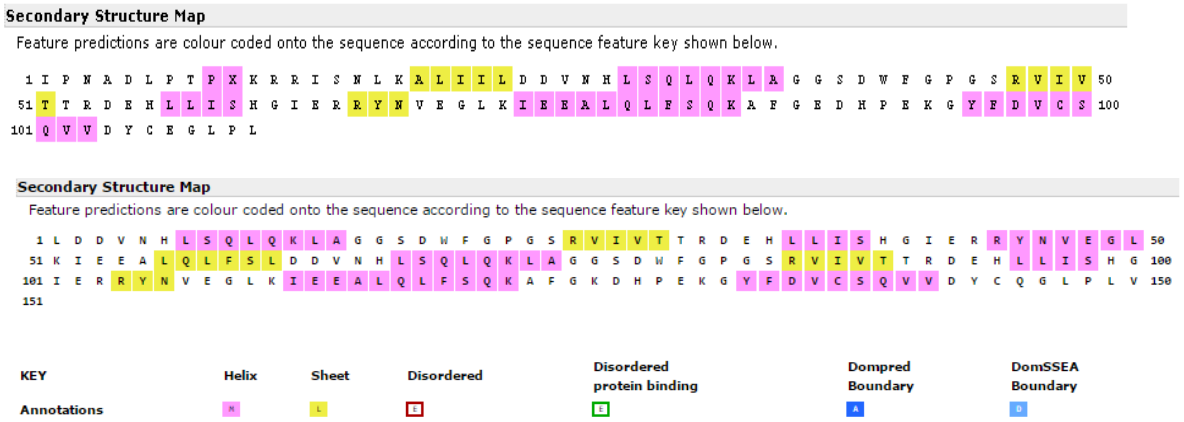

Figure 3. Secondary structure of resistance gene analogue protein 325 (Down) and 197 (Up) genes isolated from Iranian melon native type 325 and cucumber native type 197 using PSIpred.

and cloned in many plant species. However developing disease resistance is one of the most important objectives of breeding Cucurbitaceae crops because limited success has been achieved in developing cucurbit resistant cultivars due to lack of complete resistance in the cucurbits germplasm. In the current study, the first analysis of NBS-encoding genes from Iranian cucumber native types and RGHs from melon, cucumber and Cantaloupe is reported. The results contribute to the identification of candidate R-genes and provide insight into NBS-encoding gene evolution in Cucurbitaceae crops.

Using degenerate primers to isolate resistance gene analogs from different plant species has shown to be an effective strategy so far (Palomino *et al*., 2006; Bouktila *et al.,* 2014). In the present study, we identified and characterized 15 RGAs in 15 Iranian cucumber native types, using three primer combinations (Deng *et al*., 2000; Zhang *et al*., 2008). Primer set F2/R2 was the most effective, since it allowed identification of all the RGAs obtained from the Iranian cucumber native types.

The phylogenetic analysis supported the classification of Iranian cucumbers native types RGAs into TIR and non-TIR subclasses. Both TIR-NBS-LRR and non-TIR-NBS-LRR subclasses have been cloned from plants; however, the ratio of these two subclasses is variable across species. In this study, out of 15 RGAs identified, five were characterized as belonging to the TIR-NBS-LRR subclass. This result is consistent with reports in cowpea (Bhavani *et al*., 2002), lentil (Yaish *et al*., 2004), Pea (Djebbi *et al*., 2015) and soybean (Kanazin *et al*., 1996). The functional assignment of our identified RGAs shows that most of NBS-LRR genes (5/15) act as a resistance gene against *Pseudomonas syringae*. It is in agreement with other studies showing that the majority of the NBS-LRR genes provide resistance against biotrophic pathogens following a "gene-for-gene" or "guard" model of host-pathogen interaction leading to the activation of salicylic acid (SA) defense response (Glazebrook, 2005).

In addition, the phylogenetic tree revealed that the TIR *Cucumis melo* var. Cantalupensis RGAs reported here (TN-92-99) exhibited a high homology to TMV resistance protein N-like from *Cucumis melo* (XP-008463577). This suggests that the 6 Iranian cucumber native types RGAs isolated in this study might be involved in conferring resistance against either viral or bacterial pathogens. Population genetic studies showed that due to the balancing selection mechanism, NBS-LRR genes and its mutant forms widely existed in natural populations of plants (Myer *et al.,* 2005). Also it has been demonstrated that some of the NBS-LRR genes might lose function due to mutations in the absence of pathogenic stress situations in native types or in wild crops (Huang *et al*., 2009).

Result of bioinformatic analysis of candidate proteins 325 and 197 also showed that Iranian native type cucurbits have different resistance gene analogues whose expression may have an important role in response to different pathogens including *Pseudomonas syringae*. Also study on the second structure of candidate proteins 325 and 197 suggest that they have α helix structure causing them more flexible and soluble. The higher flexibility in proteins causes them to have low stability. These properties are observed in resistant proteins expressed during the stress and reaction to a pathogen (Vanlerberghe, 2013).

In 2009, the cucumber genome was sequenced by

scientist who worked on the 'Chinese Long' inbred line 9930 (Wan *et al.*, 2013), and useful information has been collected, in two cucumber genome databases (http:// cucumber.genomics.org.cn/; http://genome.jgi-psf.org/ cucumber/cucumber.home.html). However, information regarding the native type Cucurbitaceae crops is still rare, including that of Iranian native type crops. The present study identified a total of 15 RGAs in the Iranian cucumber native types RGAs, nine of which are unique sequences. The NBS-LRR genes were classified into seven distinct classes that are potentially involved in response to *Pseudomonas syringae* and TMV. The RGAs identified were tentatively characterized based on their similarities to functionally assigned sequences.

CONCLUSION

The results of this study provide a genomic framework for further isolation of candidate NBS-encoding genes in Cucurbitaceae crops through comparative genomics, and contribute to the understanding of the evolutionary mode of NBS-encoding genes in Cucurbitaceae crops. They could serve as a potential resource for future improvement and breeding of these important crops. However, the expression of these NBS–LRR genes in cultivated cucurbits should be analyzed after infection by *P. syringae* and TMV to confirm differences in disease resistance between wild type and cultivated cucurbits. Thus, obtaining more NBS sequences from these Cucurbitaceae crops should be the focus of future studies.

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