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Cloning of three nucleotide-binding site leucine-rich repeat (NBS-LRR) class resistance gene analogs in *Pistacia khinjuk*

B. Bahramnejad* and P. Shahidi

Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran.
*Corresponding author's email: b.bahramnejad@uok.ac.ir

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ABSTRACT

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***Pistacia khinjuk* (Stocks) is a native species that, along with *P. atlantica*, is widely distributed from eastern to western Iran through the Makran Zone, Zagros Mountains and the Sanandaj-Sirjan Zone, ranging from 50 to 3300 m above sea level. The identification of resistance gene analogs holds great promise for developing resistant plants. A PCR approach with degenerate primers designed from conserved nucleotide binding site-leucine rich repeat (NBS-LRR) regions of known disease resistance (R) genes was used to amplify and clone homologous sequences from *P. khinjuk*. The primers resulted in amplicons with an expected size of 500 bp. The nucleotide sequence of three amplicons was obtained through sequencing; their predicted amino acid sequences compared to each other and to the amino acid sequences of known R-genes revealed significant sequence similarity. Alignment of deduced amino acid sequence of *P. khinjuk* resistance gene analogs (RGAs) showed strong identity (42-60%) to NBS-LRR proteins R-gene subfamily from other plants. A P-loop motif (GMMGGEGKTT), conserved and hydrophobic motif GLPLAL, kinase-2a motif (LLVLDDV), where it was replaced by IAVFDDI and kinase-3a (FGPGSRIII), were present in all RGAs. A phylogenetic tree based on the deduced amino-acid sequences of *PKHRGA1, PKHRGA2, PKHRGA3,* and RGAs from different species, indicated that they were separated into two clusters The NBS analogs that we isolated can be used as guidelines to eventually isolate numerous R-genes in pistachio.**

Keywords: cloning, *Pistacia khinjuk*, resistance gene analog (RGA)

INTRODUCTION

Plants have an innate immune response, which involves a plant resistance gene (R-gene). This R-gene plays a key role in recognizing proteins expressed by specific avirulence (Avr) genes of pathogens and can defend the plant against attacks by fungi, oomycetes, bacteria, viruses, insect pests, and nematodes. These genes can be classified into five categories based on their predicted proteins (Song *et al.,* 1997; Ellis *et al.,* 2000). These genes have been identified based on specific conserved functional domains and grouped into five diverse classes (Hammond-Kosack *et al.*, 1996).

The NBS (nucleotide-binding site)-LRR (leucine-rich repeat) family is not only the most abundant class but also shows resistance to a number of pathogens. The NBS domain located at the N-terminal end contains several highly conserved motifs, such as the P-loop/kinase 1, kinase 2 and kinase 3-a, and hydrophobic GLPL. P-loop (phosphate-binding loop) is a motif in ATP- and GTP-binding proteins involved in an ATP synthase β subunit, ras protein, ribosomal elongation factor, and adenylate kinase with molecular switches (Saraste *et al.*, 1990).

Pistacia khinjuk is a native wild species of *Pistacia* widely distributed in the semi-arid and arid mountain and highland steppes throughout Iran (Sabeti, 1976). *Pistacia khinjuk* is widespread in places that are located 700-2000 meters above sea level (Aarts *et al.,* 1998). Known as Khenjuk or Kelkhong in Persian (Ghazvini *et al.,* 2007), *P. khinjuk* is an important and well-known rootstock species in pistachio nut production (Akdemir *et al*., 2013). It is also an important tree for ecological conservation and resin production in the Zagross mountain region, and its fruits are edible. *Pistacia* species have been used in folk medicine as antiflammatory, antipyretic, antibacterial, and antiviral agents and for treating diarrhea and throat infections (Aarts *et al.,* 1998; Rezaei *et al.,* 2012).

The essential oil of *P. khinjuk* has an

antihelminthic effect against protoscoleces of *Echinococcus granulosus* (Taran *et al.,* 2009). Different leaf extracts of this plant have shown antimicrobial activity against bacteria (MIC = 0.02 - 0.5 mg/ml) and fungi (MIC = 0.06 - 0.4 mg/ml) (Taran *et al.,* 2009). In Bakhtiari folk medicine, the resin of this plant is used to cure indigestion and toothaches and also as a tonic and astringent.

The PCR approach with degenerate primers is based on conserved amino acid motifs of a known NBS-LRR gene. This method has been widely used to find NBS-encoding disease resistance gene analogs (RGAs) from a variety of species, including mouse-ear cress (*Arabidopsis thaliana*) (Aarts *et al.,* 1998), tomato (Leister *et al.,* 1996), soybean (Yu *et al.,* 1996), strawberry (Zamora *et al.,* 2004), poplar (Zhang *et al.,* 2007), and wild species of peach (*Prunus kansuensis*) (Cao *et al.,* 2011). In the present study, we used a pair of degenerate oligonucleotide primers based on the NBS domain of resistance genes and obtained a new resistance gene analog from *P. khinjuk* by PCR analysis; we also compared the NBS-LRR class of RGAs obtained from *P. khinjuk* with known resistance gene sequences.

MATERIALS AND METHODS

Plant materials and DNA extraction

Seeds of *P. khinjuk* were collected from female trees in Kamyaran city, southern Kurdistan Province. Seeds were soaked in water and shaken for two days at 150 rpm. The surface layer was gently removed. Seed scarification was done as follows. Seeds were placed in a glass container and covered with sulfuric acid (30N). They were then gently stirred and allowed to soak for 10 minutes. Once the seed coat was modified (thinned), the seeds were removed, washed with distilled water and sown.

One month after seedlings emerged and were about 10 cm high, small leaves were gathered and used for DNA extraction. Total genomic DNA was isolated from the combined leaves of five *P. khinjuk* plants using the protocol described by Doyle and Doyle (1990) with the required modifications. DNA quality and quantity were determined using a WPA (Biochrom) spectrophotometer and 1% agarose gel.

Cloning and sequencing of NBS-LRR-type sequences

Resistance-gene-specific degenerate oligonucleotide primers previously used in other taxa (Joshi *et al.,* 2010) were selected. A forward primer RGAF1 was designed in sense direction, corresponding to the amino acid sequence GMGGVGKT of the NBS motif: 5′-GGNATGGGNGGNGTNGGNAA(A/G)AC-3′, and the reverse primer RGAR1 was based on the amino acid sequence GLPLALKV of the membrane-spanning motif in anti-sense direction: 5′-AC(T/C)TTNA(A/G) NGCNA(A/G)NGGNA(A/G)NCC-3′.

Reverse primer was based on motif (GLPLAL) from the *N*, *L6* and *RPS2* genes of the NBS-LRR class specific against pathogens. The primers were designed in such a way that around 500-bp PCR products were obtained upon amplification. PCR was carried out in a 25-µL reaction mixture with 20 ng template DNA, 200 nM each of the forward and reverse primers, 200 µM each of dATP, dCTP, dGTP, and dTTP, 10X PCR buffer, 15 mM MgCl2 and 1 U *Taq* DNA polymerase (Vivantis). PCR was performed in an iCycler thermal cycler (BIO RAD) using the following cyclic conditions: initial denaturation at 94°C for 5 min followed by 42 cycles each consisting of DNA denaturation at 94°C for 1 min; primer annealing at 53°C for 1 min and primer extension at 72°C for 1 min, and a final extension for 7 min. PCR products were subjected to electrophoresis on a 1.5% agarose gel in 0.5X TAE buffer, at 60 V for 1 h. The photographs of gels were taken using a gel documentation system (Biorad) for recording and analysis. PCR products were separated on 1.0% agarose gels and the expected fragments were purified from the gels using a Nucleic Acid Extraction kit (Vivantis).

The concentration of purified DNA was determined by spectrophotometer; DNA fragments were then ligated into the TA vector using a TA cloning kit (Fermentas) and transformed into competent cells of *Escherichia coli* DH5a strain. Positive clones were identified by colony PCR, and independent sequences per clone were obtained from a commercial sequencing service (Bioneer Inc. Bioneer Corporation).

Bioinformatic analysis

Sequenced fragments gave DNA sequence information with a high level of accuracy and were submitted to sequence analysis. Nucleotide sequences were translated and the corresponding amino acid sequences were aligned with NBS domain encoded by cloned R-genes using CLUSTALW software (Thompson *et al.,* 1997) to look for motifs characteristic of resistance proteins. Homology search was performed using the default settings of BLASTp with non-redundant GenBank database (http://www.ncbi.nlm.nih.gov) to identify resistance gene analogs, as well as other homologous sequences in the database. Amino acid sequences from resistance genes from other plant

species were added to the set of NBS sequences, and cluster analysis was carried out using the MEGA package based on the neighbor-joining method (Saitou and Nei, 1987).

RESULTS

Using the two R-gene specific degenerate primers, an RGA candidate was isolated from leaves of *P. khinjuk*. Genomic DNA was isolated efficiently from leaves of young *P. khinjuk* seedlings (Fig. 1a). PCR amplification with genomic DNA resulted in the production of amplicons of the predicted size (about 500 bp) based on previously published RGA sequences (Fig. 1b). The amplicons were cloned and 10 colonies were sequenced. Following homology searches using the BLASTX algorithm, the sequenced fragments showed significant homology with the NBS domain of known R-genes or RGCs cloned from other plant species (Table 1) and designated as *PKHRGA1, PHKRGA2* and *PKHRGA3*. The *PAKRGAs* sequences showed a high level of sequence identity to comparable regions of disease resistance genes published in GenBank, supported by low e-values.

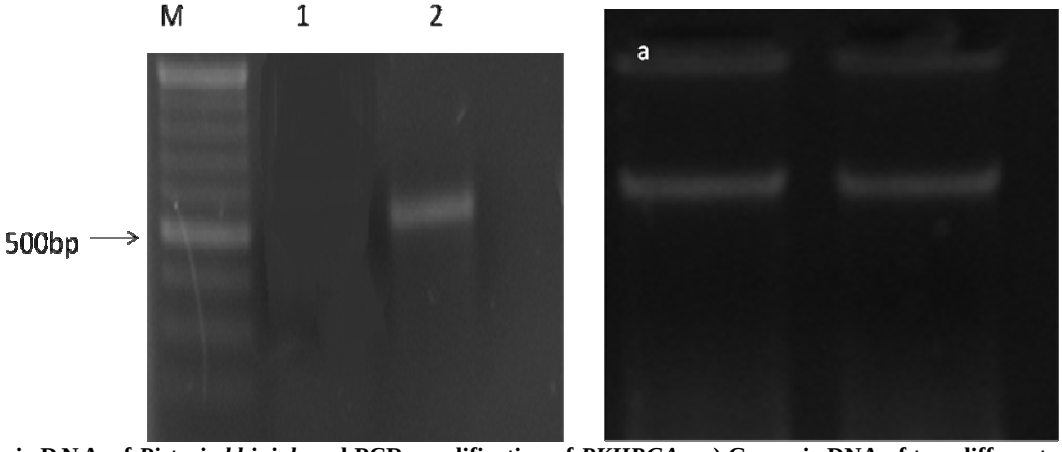
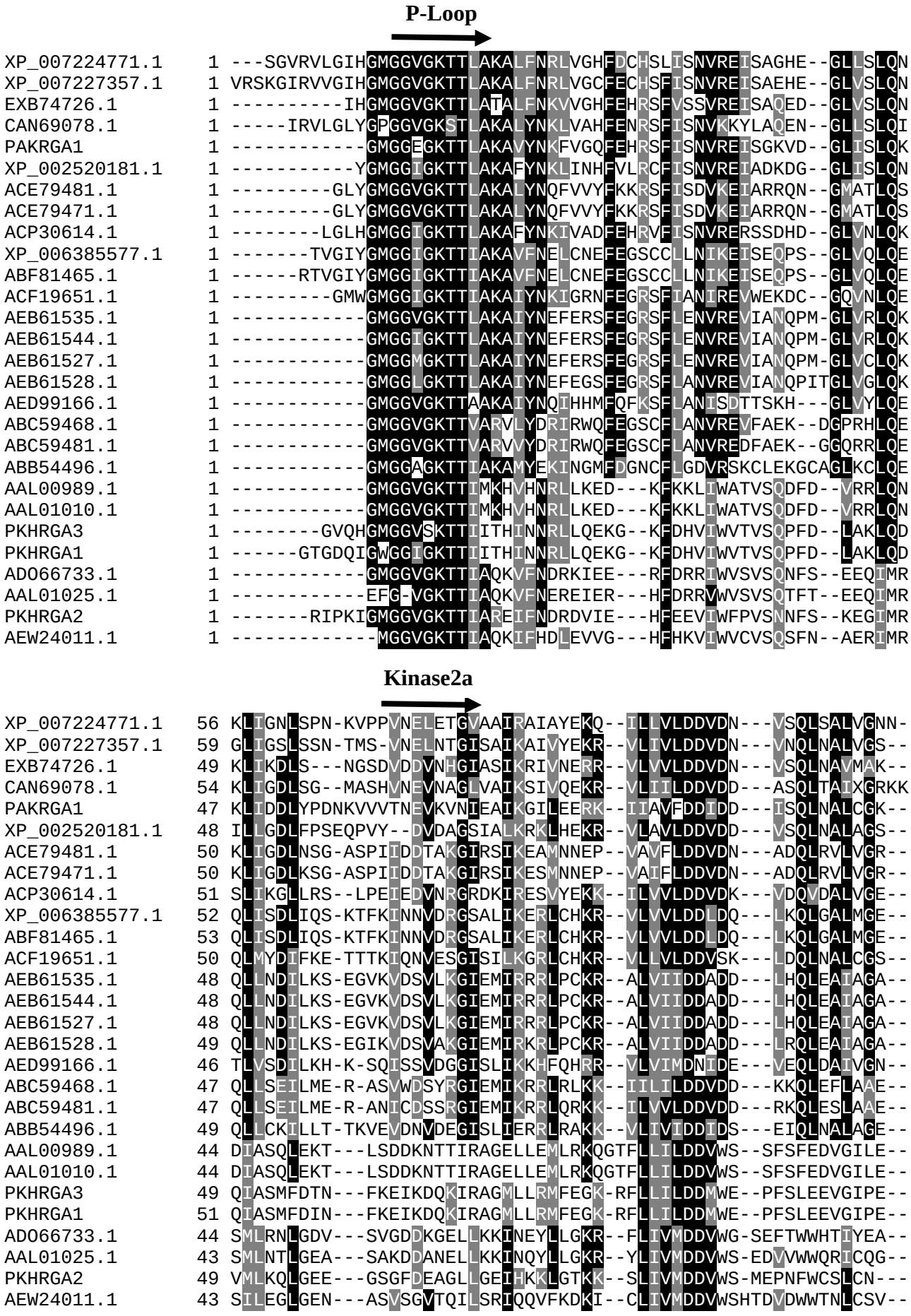


Fig. 1. Genomic DNA of *Pistacia khinjuk* and PCR amplification of *PKHRGAs*. a) Genomic DNA of two different samples from young seedlings. b) PCR amplification of *PKHRGAs* using the combined degenerate primers designed according to the conserved NBS-LRR motif of several plant R genes. M is the 2-kb DNA ladder; 1 is negative control; 2 is a *P. khinjuk* PCR product.

Blastp searches of deduced amino acid sequences of the *PKHRGAs* revealed the presence of NBS domain and significant homology to well-characterized R genes from angiosperms. *PKHRGA1* and *PHKRGA3* showed 92.8% identity, while *PKHRGA2* showed much lower identity with *PKHRGA1* and *PHKRGA3*. The identities of *PKHRGA2* with *PKHRGA1* and *PHKRGA3* were 24.39% and 23.03%, respectively. The 3' end of *PKHRGA2* was very different from *PKHRGA1* and *PHKRGA3*.

Multiple alignments of the deduced amino acid sequences of *PKHRGA1, PKHRGA2* and *PHKRGA3*, along with the selected RGA sequences from other plants, revealed the presence of the conserved resistance P-loop motif (GMGGVGKT) (Fig. 2). In addition, motif kinase-2a (LLVLDDV), kinase-3a (FGPGSR) and GLPL motifs were also present in *PKHRGA1* and *PHKRGA3*. The analysis showed that in all three gene motifs, P-loop and kinase-3a were highly conserved, but motif kinase-2a was relatively less conserved. Motif GLPL is not present in *PKHRGA2*. Analyses of the nucleotide polymorphism and diversity of *P. khinjuk* and other RGAs showed that they were highly conserved at the P-loop and comparatively more conserved at II, Kinase-2a, Kinase-3a, and GLPL motifs than at other parts of the sequences. This high conservation at the P-loop and GLPL motifs of RGAs may due to degenerate primer sequences (Table 2).

To estimate the phylogenetic relationship among *PKHRGAs* and other sequences of known plant NBS encoding R genes, a neighbor-joining phylogenetic tree was constructed. From the phylogenetic tree, genetic divergence between of RGA sequences was observed. Sequences were classified into two clusters (Fig. 3). *PKHRGA1, PHKRGA2* and *PKHRGA3* were classified into class II along with RGAs. Cluster II consisted of two subclusters. *PKHRGA1* and *PKHRGA3* were in a subcluster with AAL00989.1 and AAL01010.1 (*Theobroma cacao*), AAO89145.1, AAO89146.1 and AAO89151.1 (*Gossypium barbadense*), AFC90831.1 (*Rhododendron formosanum*) and AGM46232.1 (*Gossypium hirsutum*). *PHKRGA2* was in another subcluster with AEW24011.1 (*Rubus* sp.), AAL01025.1 (*Theobroma cacao*), ADO66733.1 (*Prunus kansuensis*), ADA59480.1


P-Loop
Kinase2a

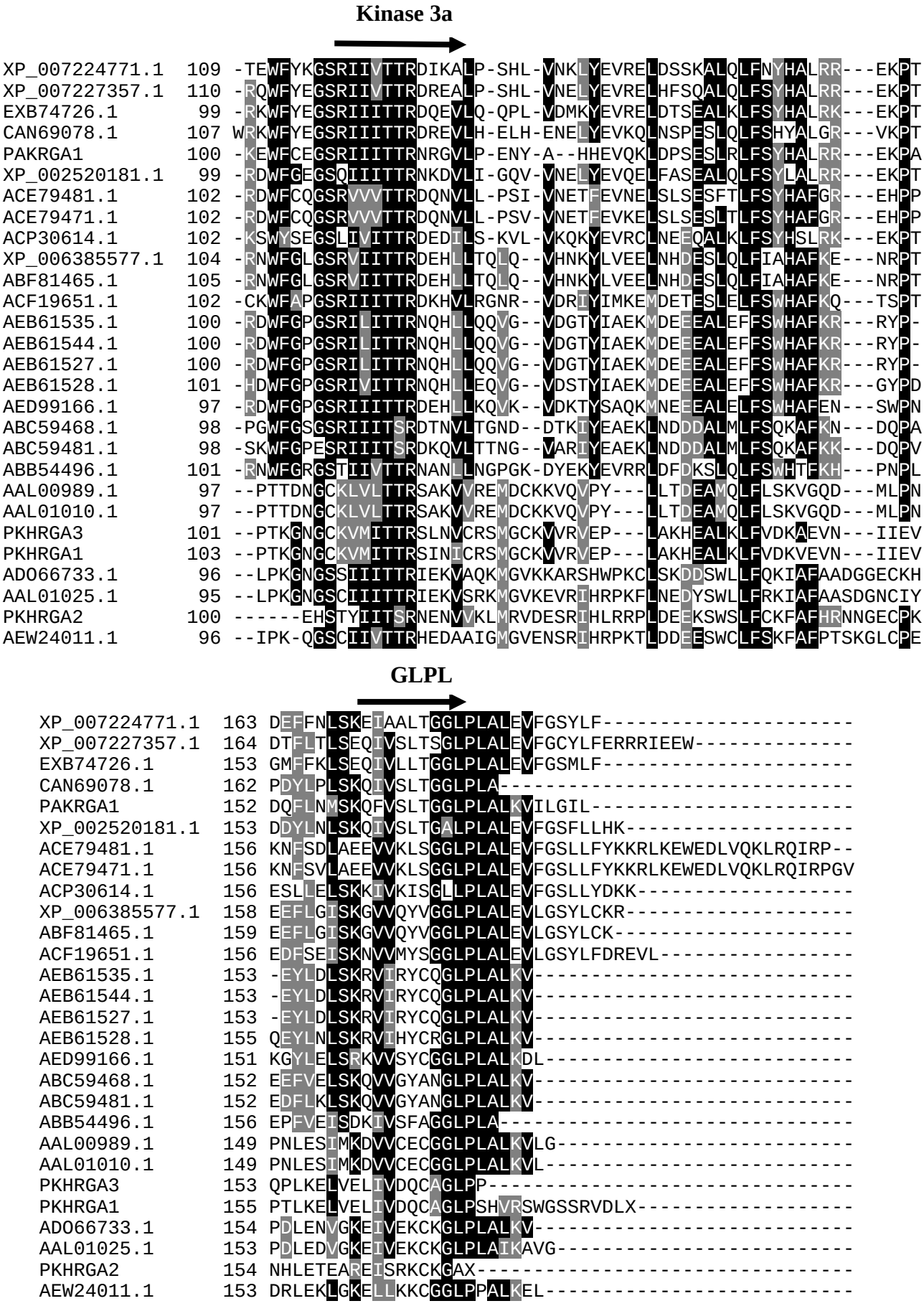


Fig. 2. Multiple alignments of the consensus amino acid sequences of the 21 RGAs and NBS domain of R-genes along with PKHRGAs of *Pistacia khinjuk* constructed with Clustal W. Conserved motifs are numbered as in Lescot *et al.* (2004); P-loop, Kinase-2a, Kinase-3a, RNBS-C and GLPL motifs.

Table 1. Sequence homology comparisons of *PKHRGAs* with highest similarity sequences, expected value and identity percentage.

Gene	Accession	Description	E value	Identity
PKHRGA1,	**XP_006465222.1**	**probable disease resistance protein At1g61310-like [*Citrus sinensis*]**	**2e-54**	**60%**
PKHRGA3	**XP_006465217.1**	**probable disease resistance protein At5g63020-like [*Citrus sinensis*]**	**6e-52**	**60%**
	XP_006465218.1	**disease resistance protein At4g27190-like [*Citrus sinensis*]**	**1e-51**	**59%**
	XP_006465216.1	**disease resistance protein At4g27190-like [*Citrus sinensis*]**	**4e-50**	**58%**
	XP_006465223.1	**probable disease resistance protein At5g43730-like [*Citrus sinensis*]**	**3e-48**	**56%**
	XP_006493390.1	**probable disease resistance protein At5g43740-like [*Citrus sinensis*]**	**3e-47**	**56%**
	XP_006465219.1	**putative disease resistance protein At4g10780-like [*Citrus sinensis*]**	**4e-45**	**54%**
	AFC90617.1	**nucleotide-binding site leucine-rich repeat protein [*Rhododendron formosanum*]**	**4e-45**	**49%**
	XP_006465215.1	**disease resistance protein RPS5-like [*Citrus sinensis*]**	**5e-45**	**54%**
	AGM46232.1	**NBS-LRR resistance protein-like protein [*Gossypium hirsutum*]**	**8e-45**	**48%**
PKHRGA2	**XP_006487863.1**	**disease resistance RPP13-like protein 4-like [*Citrus sinensis*]**	**8e-49**	**53%**
	XP_006487739.1	**disease resistance RPP13-like protein 4-like [*Citrus sinensis*]**	**4e-48**	**53%**
	ADA59480.1	**NBS-coding resistance protein-like protein RGA8 [*Solanum aculeatissimum*]**	**4e-41**	**48%**
	ABC59507.1	**NBS-LRR disease resistance-like protein [(*Populus tomentosa x P. bolleana*) x *P. tomentosa* var. truncata]**	**2e-39**	**46%**
	AEV76897.1	**resistance protein RGA14 [*Capsicum annuum*]**	**2e-39**	**48%**
	AEW24011.1	**putative NBS-LRR disease resistance protein [*Rubus* sp. LAK-2011]**	**3e-39**	**46%**
	ADO66733.1	**NBS-LRR-like protein [*Prunus kansuensis*]**	**9e-39**	**47%**
	AEV76898.1	**resistance protein RGA15 [*Capsicum annuum*]**	**1e-38**	**47%**
	XP_006360547.1	**disease resistance RPP13-like protein 4-like [*Solanum tuberosum*]**	**3e-38**	**47%**
	XP_004243430.1	**disease resistance RPP13-like protein 4-like [*Solanum lycopersicum*]**	**7e-38**	**47%**
	EXB41593.1	**Disease resistance RPP13-like protein 4 [*Morus notabilis*]**	**1e-37**	**42%**
	ACJ05260.1	**NBS-LRR protein [*Pyrus sinkiangensis*]**	**3e-37**	**46%**

Table 2. Resistance gene analogs (RGAs) amino acid sequences used in this study.

Accession number	Gene description	Species	Length (AA)
AAL00989.1	**NBS/LRR resistance protein-like protein**	***Theobroma cacao***	**173**
AAL01010.1	**NBS/LRR resistance protein-like protein**	***Theobroma cacao***	**172**
AAO89145.1	**NBS/LRR resistance protein-like protein**	***Gossypium barbadense***	**166**
AAO89146.1	**NBS/LRR resistance protein-like protein**	***Gossypium barbadense***	**166**
AAO89151.1	**NBS/LRR resistance protein-like protein**	***Gossypium barbadense***	**166**
AFC90831.1	**nucleotide-binding site leucine-rich repeat protein**	***Rhododendron formosanum***	**262**
AGM46232.1	**NBS-LRR resistance protein-like protein**	***Gossypium hirsutum***	**168**
AEB61535	**NBS resistance protein**	***Prunus persica***	**174**
AEB61544	**NBS resistance protein**	***Prunus persica***	**174**
AEB61527	**NBS resistance protein**	***Prunus persica***	**174**
AEB61528	**NBS resistance protein**	***Prunus persica***	**174**
ACJ05252.1	**NBS leucine-rich repeat disease resistance**	***Pyrus pyrifolia***	**168**
AED99166.1	**NBS-LRR-like protein**	***Malus baccata***	**174**
ACF19651.1	**TIR-NBS-LRR RCT1-like resistance protein**	***Medicago sativa***	**1125**
ABB54496.1	**RSP-1**	***Ipomoea batatas***	**175**
ABC59468.1	**NBS-LRR disease resistance**	***Populus hybrid***	**174**
ABC59481.1	**NBS-LRR disease resistance**	***Populus hybrid***	**174**
XP_006385577.1	**NBS-LRR predicted**	***Populus trichocarpa***	**1141**
ABF81465.1	**TIR-NBS-LRR type disease resistance**	***Populus trichocarpa***	**1139**
ACP30614.1	**disease resistance protein**	***Brassica rapa subsp. pekinensis***	**1309**
ACE79481.1	**NBS-coding resistance gene analog**	***Nicotiana sylvestris***	**265**
ACE79471.1	**NBS-coding resistance gene analog**	***Nicotiana tabacum***	**267**
EXB74726.1	**TMV resistance protein N**	***Morus notabilis***	**824**
XP_007224771.1	**hypothetical protein PRUPE_ppa024045mg**	***Prunus persica***	**1372**
ADV31388.1	**nucleotide binding site**	***Citrus reticulata x Citrus trifoliata].***	**171**
Current study (PKRGA1)	**RGA candidate**	***Pistacia khinjuk***	**171**
Current study(PKRGA2)	**RGA candidate**	***Pistacia khinjuk***	**171**
Current study(PKRGA3)	**RGA candidate**	***Pistacia khinjuk***	**187**
PAKRGA1	**RGA candidate**	***Pistacia atlantca* subsp. *kurdica***	**171**
CAN69078.1	**Hypotical protein**	***Vitis vinifera***	**1478**
XP_002520181.1	**leucine-rich repeat-containing protein**	***Ricinus communis***	**619**
AEV76897.1	**resistance protein RGA14**	***Capsicum annuum***	**172**
ADA59480.1	**NBS-coding resistance protein-like protein RGA8**	***Solanum aculeatissimum***	**172**
ADO66733.1	**NBS-LRR-like protein**	***Prunus kansuensis***	**176**
AAL01025.1	**NBS/LRR resistance protein-like protein,**	***Theobroma cacao***	**255**
AEW24011.1	**putative NBS-LRR disease resistance**	***Rubus* sp.**	**176**

(*Solanum aculeatissimum*), and AEV76897.1 and AEV76898 (*Capsicum annuum*).

DISCUSSION

PCR amplification with degenerate oligonucleotide primers is a sensitive and efficient method for cloning RGAs that are potential candidates for functional resistance genes (Yu *et al.,* 1996). We isolated three genomic RGAs of the NBS-LRR type from *P. khinjuk*. The PCR-derived sequence was identified as RGA based on the following features: high sequence identities to known R genes/RGAs from other species; presence of conserved motifs characteristic of NBS-LRR R genes and uninterrupted open reading frames (ORFs) of considerable length (Deng *et al.,* 2000). The NBS sequences identified in other plant species with a similar approach showed a comparable range of identities to *PKHRGA1 PKHRGA2* and *PKHRGA3.*

In *PKHRGA1*, PKHRGA2 and *PKHRGA3*, P-loop was very similar to other RGAs, while kinase

domains were very variable. The last residue of kinase-2 motif can be used to predict a subclass of NBS–LRR R-genes with 95% accuracy

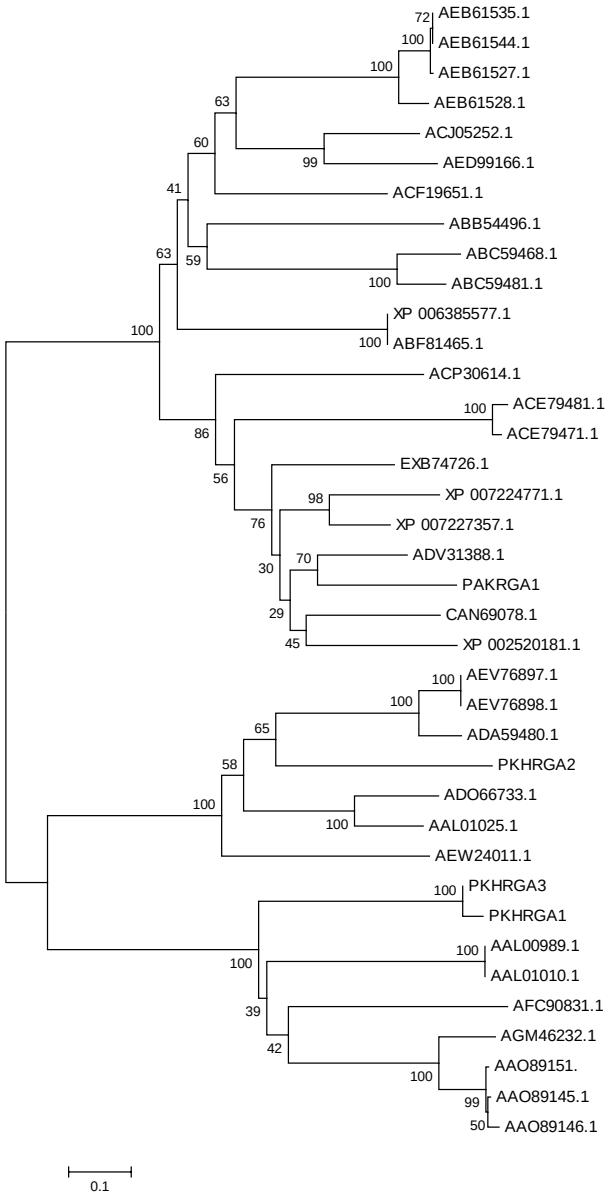


Fig. 3. Consensus tree of NBS encoding RGAs in *Pistacia khinjuk* constructed by phylogenetic analysis using the Clustal W program. Numbers next to the nodes give bootstrap values. The tree comprises previously reported RGAs sequences and *P. khinjuk* (Table 2).

(Meyers *et al.,* 1999). A tryptophan residue (W) is expected at the end of kinase-2 motif in non-TIR NBS-LRR sequences, while an aspartic acid (D) or asparagine (N) residue is expected for TIR NBS–LRR sequences. Using this criterion, it was observed that all *PKHRGAs* belonged to the non-TIR NBS–LRR subclass.

Phylogenetic analysis of NBS analogs identified their group based on similarity. The phylogenetic tree based on neighbor joining using % identity of the deduced amino acid sequences of *P. khinjuk* and other species identified their relatedness with known R-genes. The phenetic tree classified the RGAs in two classes. Similar results have been reported previously (Joshi *et al.*, 2010). Three isolated RGAs were in different places in the phylogenetic tree. *PKHRGA1* and *PKHRGA3* were very close together, while *PKHRGA2* was in another sub-cluster. The 3' end of PKHRGA2 was different from the other two RGAs. This may due to frame shift mutation which changed the reading frame. However, isolation, sequencing and analysis of more and more *Pistacia* NBS analogs are required to gain better knowledge about the RGAs and draw further conclusions.

Iran is a center of origin for four important *Pistacia* species: *P. vera* L., *P. khinjuk* Stocks, *P. eurycarpa* Yalt. (*P. atlantica* subsp. *kurdica* Zoh.) and *P. atlantica* Dsef. (Karimi and Kafkas, 2011). *Pistacia khinjuk* and *P. atlantica* are the rootstocks most resistant to root-knot nematode and drought. *Pistacia* species have high genetic diversity due to their dioecious character, pollination mechanism and high heterozygosity (Karimi and Kafkas, 2011). Therefore, it is possible that *P. khinjuk* may have a large number of resistance genes. With the help of the identified *P. khinjuk* RGAs, different primer sets can be designed for analysis of *Pistacia* wild relatives to target novel genomic resources for the genetic improvement of *Pistacia*.

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