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

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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. 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 (leucinerich repeat) family is not only the most abundant class but also shows resistance to a number of pathogens. The NBS domain located at the Nterminal 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 membranespanning 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-uL 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 encoded by cloned R-genes using domain 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 PKHRGA1, as 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 wellcharacterized 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 PKHRGA1. PKHRGA2 of 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 AFC90831.1 barbadense), (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

XP_007224771.1	1	SGVRVLGIH <mark>GMGGVGKTTLAKALFN</mark> RLVGHFDCHSLIS <mark>NVRE</mark> ISAGHEGLLSLQN
XP_007227357.1	1	VRSKGIRVVGIH <mark>GMGGVGKTTLAKA</mark> LFNRLVGC <mark>FE</mark> CHS <mark>FIS</mark> NVREISAEHEGLVSLQN
EXB74726.1	1	IH <mark>GMGGVGKTTLAT</mark> ALFNKVVGH <mark>FE</mark> HRSFVSSVREISAQEDGLVSLQN
CAN69078.1	1	IRVLGLY <mark>GPGGVGKSTLAKALYNKLVAH</mark> FE <mark>NRSFISNVK</mark> KYLAQEN <mark>GLLS</mark> LQI
PAKRGA1	1	GMGG <mark>E</mark> GKTTLAKAVYNKFVGQFEHRSFISNVREISGKVDGLISLQK
XP_002520181.1	1	Y <mark>GMGGICKTTLAKA</mark> FYNKLINH <mark>FVLRC</mark> FIS <mark>NVREI</mark> ADKDGCLISLQN
ACE79481.1	1	GLY <mark>GMGGVGKTTLAKALYN</mark> QFVVY <mark>F</mark> KKRSFISDVKEIARRQNCMATLQS
ACE79471.1	1	GLY <mark>GMGGVGKTTLAKALYN</mark> QFVVY <mark>F</mark> KKRS <mark>FI</mark> SDVKEIARRQNCMAT <mark>LQ</mark> S
ACP30614.1	1	LGLH <mark>GMGGIGKTTLAKA</mark> FYNKIVAD <mark>FE</mark> HRVFISNVRERSSDHDGLVNLQK
XP_006385577.1	1	TVGIY <mark>GMGGIGKTTIAKA</mark> VFN <mark>ELCNEFE</mark> GSCCLLNIKEISEQPSGLVQLQE
ABF81465.1	1	RTVGIY <mark>GMGGIGKTTIAKA</mark> VFN <mark>E</mark> LCNE <mark>FE</mark> GSCCLLNIKEISEQPSGLVQLQE
ACF19651.1	1	GMWGMGGIGKTTIAKAIYNKIGRNFEGRSFIANIREVWEKDCGQVNLQE
AEB61535.1	1	<mark>GMGGVGKTTLAKAIYN</mark> EFERS <mark>FE</mark> GRSFLE <mark>NVREVIA</mark> NQPM- <mark>GLVRLQ</mark> K
AEB61544.1	1	<mark>GMGGIGKTTLAKAIYN</mark> EFERS <mark>FE</mark> GRSFLE <mark>NVREVIA</mark> NQPM- <mark>GLVRLQ</mark> K
AEB61527.1	1	GMGGMGKTTLAKAIYNEFERS <mark>FE</mark> GRS <mark>FLE</mark> NVREVIANQPM-GLVC <mark>LQ</mark> K
AEB61528.1	1	GMGGLGKTTLAKAIYNEFEGS <mark>FE</mark> GR <mark>S</mark> FLANVREVIANQPITGLVGLQK
AED99166.1	1	<mark>GMGGVGKTT</mark> AAKAIYNQIHHMFQFKSFLANISDTTSKHGLVYLQE
ABC59468.1	1	<mark>GMGGVGKTTVARVLY</mark> DRIRWQ <mark>FE</mark> GSC <mark>FLANVRE</mark> VFAEKD <mark>G</mark> PRHLQE
ABC59481.1	1	<mark>GMGGVGKTTVARVVY</mark> DRIRWQ <mark>FE</mark> GSC <mark>FLANVRE</mark> DFAEKG <mark>G</mark> QRRLQE
ABB54496.1	1	GMGGAGKTTIAKAMYEKINGMFDGNCFLGDVRSKCLEKGCAGLKCLQE
AAL00989.1	1	KFKKLIWATVSQDFDVRRLQN
AAL01010.1	1	KFKKLIWATVSQDFDVRRLQN
PKHRGA3	1	GVQH <mark>GMGGV</mark> SKTTIITHINNRLLQEKGKFDHVIWVTVSQPFDLAKLQI
PKHRGA1	1	GTGDQI <mark>GWGGIGKTTI</mark> ITHINNRLLQEKGK <mark>F</mark> DHVIWVTVSQPFDLAKLQI
AD066733.1	1	R <mark>F</mark> DRRIWVSVSQNFSEEQIMF
AAL01025.1	1	HFDRRWWVSVSQTFTEEQIMF
PKHRGA2	1	RIPKI <mark>GMGGVGKTTIARE</mark> IFNDRDVIEH <mark>F</mark> EEVIWFPVSNNFSKEGIMF
AEW24011.1	1	HFHKVIWVCVSQSFNAERIMF

Kinase2a

XP_007224771.1	56	KLIGNLSPN-KVPPVNELETGVAAIRAIAYEKQILLVLDDVDNVSQLSALVCNN-
XP_007227357.1	59	GLIGSLSSN-TMS-VNELNTGISAIKAIVYEKRVLIVLDDVDNVNQLNALVGS
EXB74726.1	49	KLIKDLSNGSDVDDVNHGIASIKRIVNERRVLVVLDDVDNVSQLNAVMAK
CAN69078.1	54	KLIGDLSGMASHVNEVNAGLVAIKSIVQEKRVLIILDDVDDASQLTAIXCRKK
PAKRGA1	47	KLIDDLYPDNKVVVTNEVKVNIEAIKGILEERKIIAVFDDIDDISQLNALCCK
XP_002520181.1	48	ILLGDLFPSEQPVYDVDAGSIALKRKLHEKRVLAVLDDVDDVSQLNALAGS
ACE79481.1	50	KLIGDLNSG-ASPIIDDTAKGIRSIKEAMNNEPVAVFLDDVDNADQLRVLVCR
ACE79471.1	50	KLIGDLKSG-ASPIIDDTAKGIRSIKESMNNEPVAIFLDDVDNADQLRVLVCR
ACP30614.1	51	SLIKGLLRSLPEIEDVNRGRDKIRESVYEKKILVVLDDVDKVDQVDALVGE
XP_006385577.1	52	QLISDLIQS-KTFKINNWDRGSALIKERLCHKRVLVVLDDLDQLKQLGALMGE
ABF81465.1	53	QLISDLIQS-KTFKINN <mark>V</mark> DRGSALIKERLCHKRVLVVLDDLDQLKQLGALMGE
ACF19651.1	50	QLMYDIFKE-TTTKIQNVESGISILKGRLCHKRVLLVLDDVSKLDQLNALCGS
AEB61535.1	48	QLLNDILKS-EGVKVDS <mark>V</mark> LKGIEMIRRRLPCKRALVIIDDADDLHQLEAIAGA
AEB61544.1	48	QLLNDILKS-EGVKVDS <mark>V</mark> LKGIEMIRRRLPCKRALVIIDDADDLHQLEAIACA
AEB61527.1	48	QLLNDILKS-EGVKVDS <mark>V</mark> LKGIEMIRRRLPCKRALVIIDDADDLHQLEAIAGA
AEB61528.1	49	QLINDILKS-EGIKVDSWAKGIEMIRKRLPCKRALVIIDDADDLRQLEAIAGA
AED99166.1	46	TLVSDILKH-K-SQISS <mark>V</mark> DG <mark>GI</mark> SLIKKHFQHRRVLVIMDNIDEVEQLDAIVCN
ABC59468.1	47	QLLSEILME-R-ASVWDSYRGIEMIKRRLRLKKIIILILDDVDDKKQLEFLAAE
ABC59481.1	47	QLLSEILME-R-ANICDSSRGIEMIKRRLQRKKILVVLDDVDDRKQLESLAAE
ABB54496.1	49	QLLCKILLT-TKVEVDNVDEGISLIERRLRAKKVLIVIDDIDSEIQINALAGE
AAL00989.1	44	DIASQLEKTLSDDKNTTIRAGELLEMLRKQGTFLLILDDVWSSFSFEDVGILE
AAL01010.1	44	DIASQUEKTLSDDKNTTIRAGELLEMLRKQGTFLLILDDVWSSFSFEDVGILE
pkhrga3	49	QIASMFDTNFKEIKDQKIRAGMLLRMFEGK-RFLLILDDMWEPFSLEEVGIPE
PKHRGA1	51	QIASMFDINFKEIKDQKIRAGMLLRMFEGK-RFLLILDDMWEPFSLEEVGIPE
AD066733.1	44	SMLRNLGDVSVGDDKGELLKKLNEYLLGKRFLIVMDDVWG-SEFTWWHTIYEA
AAL01025.1	43	SMLNTLGEASAKDDANELLKKINQYLLGKRYLIVMDDVWS-EDVVWWQRICQG
PKHRGA2	49	VMLKQLGEEGSGFDEAGLLGELHKKLGTKKSLIVMDDVWS-MEPNFWCSLCN
AEW24011.1	43	SILEGIGENASVSGVTQIISRIQQVFKDKICLIVMDDVWSHTDVDWWTNLCSV

Kinase 3a

XP_007224771.1	109 -TEWFYKGSRIIVTTRDIKALP-SHL-VNKLYEVRELDSSKALQLENYHALRREKP	г
XP_007227357.1	110 -RQWFYEGSRIIVTTRDREALP-SHL-VNELYEVRELHFSQALQLFSYHALRREKP	Г
EXB74726.1	99 -RK <mark>WFYEGSRIIITTR</mark> DQEVLQ-QPL-VDMKYEVRELDTSEALKLFSYHALRKEKP	Г
CAN69078.1	107 WRKWFYE <mark>GSRIIITTR</mark> DREVLH-ELH-ENELYEVKQLNSPESLQLFSHYALGRVKP	Г
PAKRGA1	100 -KEWFCEGSRIIITTRNRGVLP-ENY-AHHEVQKLDPSESLRLFSYHALRREKP	A
XP_002520181.1	99 -R <mark>DWFG</mark> EGSQIIIITTRNKDVLI-GQV-VNELYEVQELFASEALQLFSYLALRREKP	Г
ACE79481.1	102 -RDWFCQGSRVVVTTRDQNVLL-PSI-VNETFEVNELSLSESFTLFSYHAFGREHP	Ρ
ACE79471.1	102 -RDWFCQGSRVVVTTRDQNVLL-PSV-VNETFEVKELSLSESLTLFSYHAFGREHP	Ρ
ACP30614.1	102 -KSWYSEGSLIVITTRDEDILS-KVL-VKQKYEVRCLNEEQALKLFSYHSLRKEKP	г
XP_006385577.1	104 -RNWFGLGSRVIITTRDEHLLTQLQWHNKYLVEELNHDESLQLFIAHAFKENRP	Г
ABF81465.1	105 -RNWFGLGSRVIITTRDEHLLTQLQWHNKYLVEELNHDESLQLFIAHAFKENRP	г
ACF19651.1	102 -CKWFAPGSRIIITTRDKHVLRGNRVDRIYIMKEMDETESLELFSWHAFKQTSP	Г
AEB61535.1	100 -RDWFGPGSRILITTRNQHLLQQVGVDGTYIAEKMDEEEALEFFSWHAFKRRYP	-
AEB61544.1	100 -RDWFGPGSRILITTRNQHLLQQVGVDGTYIAEKMDEEEALEFFSWHAFKRRYP	-
AEB61527.1	100 -RDWFGPGSRILITTRNQHLLQQVGVDGTYIAEKMDEEEALEFFSWHAFKRRYP	-
AEB61528.1	101 -HDWFGPGSRIVITTRNQHLLEQVGVDSTYIAEKMDEEEALEFFSWHAFKRGYP	D
AED99166.1	97 -RD <mark>WFG</mark> P <mark>GSRIIITTR</mark> DEHLLKQVKVDKTYSAQKMNEEEALELFSWHAFENSWPI	Ν
ABC59468.1	98 -PG <mark>WFG</mark> S <mark>GSRIIIT</mark> SRDTN <mark>VL</mark> TGNDDTKIYEAEKLNDDDALMLFSQKAFKNDQP	A
ABC59481.1	98 -SKWFGPESRIIITSRDKQVLTTNGVARIYEAEKLNDDDALMLFSQKAFKKDQP	V
ABB54496.1	101 -RNWFGRGSTIIVTTRNANLLNGPGK-DYEKYEVRRLDFDKSLQLFSWHTFKHPNP	L
AAL00989.1	97PTTDNGCKLVLTTRSAKVVREMDCKKVQVPYLLTDEAMQLFLSKVGQDMLP	Ν
AAL01010.1	97PTTDNGCKLVLTTRSAKVVREMDCKKVQVPYLLTDEAMQLFLSKVGQDMLP	Ν
PKHRGA3	101 PTKGNGCKVMITTRSLNVCRSMGCKVVRVEPLAKHEALKLEVDKAEVNIIE	V
PKHRGA1	103 PTKGNGCKVMITTRSINICRSMGCKWVRVEPLAKHEALKLEVDKVEVNIIE	V
AD066733.1	96LPKGNGSSIIIITTRIEKVAQKMGVKKARSHWPKCLSKDDSWLLFQKIAFAADGGECK	Н
AAL01025.1	95LPK <mark>G</mark> N <mark>GS</mark> CIIITTRIEKVSRKMGVKEVRIHRPKFLNEDYSWLLFRKIAFAASDGNCI	Y
PKHRGA2	100EHSTYIITSRNENVVKLMRVDESRIHLRRPLDEEKSWSLFCKFAFHRNNGECP	Χ
AEW24011.1	96IPK-Q CS C IIVTTR HEDAAIGMGVENSRIHRPKTLDDEESWCLFSKFAFPTSKGLCP	E

GLPL

XP_007224771.1	163	DEFFN <mark>LSK</mark> EIAALT <mark>GGLPLAL</mark> EVFGSYLF
XP_007227357.1	164	DTFLTLSEQIVSLTSGLPLALEVFGCYLFERRRIEEW
EXB74726.1	153	GMFFK <mark>LSEQIV</mark> LLT <mark>GGLPLAL</mark> EVFGSMLF
CAN69078.1	162	PDYLPLSKQIVSLTGGLPLA
PAKRGA1	152	DQFLNMSKQFVSLTGGLPLALKVILGIL
XP_002520181.1	153	DDYLNLSKQIVSLTGALPLALEVFGSFLLHK
ACE79481.1	156	KNFSDLAEEVVKLSGGLPLALEVFGSLLFYKKRLKEWEDLVQKLRQIRP
ACE79471.1	156	KNFSVLAEEVVKLSGGLPLALEVFGSLLFYKKRLKEWEDLVQKLRQIRPGV
ACP30614.1	156	ESLLE <mark>LSK</mark> KIVKIS <mark>G</mark> LLPLALEVFGSLLYDKK
XP_006385577.1	158	EEFLGISKGVVQYVGGLPLALEVLGSYLCKR
ABF81465.1	159	EEFLGI <mark>SK</mark> GVVQYV <mark>GGLPLAL</mark> EVLGSYLCK
ACF19651.1	156	EDF <mark>SEISK</mark> NVVMYS <mark>GGLPLAL</mark> EVLGSYLFDREVL
AEB61535.1	153	-EYLD <mark>LSK</mark> RVIRYCQ <mark>GLPLAL</mark> KV
AEB61544.1	153	-EYL <mark>DLSKR</mark> VIRYCQ <mark>GLPLAL</mark> KV
AEB61527.1	153	-EYLD <mark>LSK</mark> RVIRYCQ <mark>GLPLAL</mark> KV
AEB61528.1	155	QEYLNLSKRVIHYCRGLPLALKV
AED99166.1	151	KGYLELSRKVVSYC <mark>GGLPLAL</mark> KDL
ABC59468.1	152	EEFVE <mark>LSKQVV</mark> GYAN <mark>GLPLAL</mark> KV
ABC59481.1	152	EDFLKLSKQVVGYANGLPLALKV
ABB54496.1	156	EPFVEISDKIVSFAGGLPLA
AAL00989.1	149	PNLESI <mark>MK</mark> DVVCEC <mark>GGLPLAL</mark> KVLG
AAL01010.1	149	PNLESIMKDVVCECGGLPLALKVL
PKHRGA3	153	QPLKE <mark>L</mark> VELIVDQCAGLPP
PKHRGA1	155	PTLKELVELIVDQCAGLPSHVRSWGSSRVDLX
AD066733.1	154	PDLENVG <mark>KEIV</mark> EKCK <mark>GLPLAL</mark> KV
AAL01025.1	153	PDLEDVG <mark>K</mark> EIVEKCK <mark>GLPLA</mark> IKAVG
PKHRGA2	154	NHLETEAREISRKCKCAX
AEW24011.1	153	DRLEK <mark>I</mark> G <mark>K</mark> ELLKKC <mark>GGLP</mark> P <mark>AL</mark> KEL

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.

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	Table 1. S	Sequence homology	comparisons of I	P <i>KHRGAs</i> with high	est similarity sec	uences, exp	pected value an	nd identity	percentage
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le 1. Sequence	nomology comparis	sons of <i>PKHRGAs</i> with highest similarity sequences, expected value and iden	tity percenta	ge.
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%
	XP 006487863.1	disease resistance RPP13-like protein 4-like [Citrus sinensis]	8e-49	53%
PKHRGA2	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)	2e-39	46%
		x P. tomentosa var. truncata]		
	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	171
		Citrus trifoliata].	
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, Ploop 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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