Euphytica (2015) 205:903–913
DOI 10.1007/s10681-015-1438-8

Cloning and functional analyses of pepper *CaRKNR* involved in *Meloidogyne incognita* resistance

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Received: 26 May 2014 / Accepted: 30 March 2015 / Published online: 9 April 2015

Abstract Root-knot nematodes (*Meloidogyne* spp.) are destructive pests of crops. Pepper (*Capsicum annuum* L.) contains genes that control resistance to root-knot nematodes. Using suppression subtractive hybridization and RACE strategies, a nucleotide-binding site and leucine-rich repeat (NBS-LRR) family gene, *CaRKNR* (FJ231739), was isolated and cloned from the nematode-resistant pepper line HDA149. *CaRKNR* is a novel NBS-LRR gene with an open reading frame of 3600 bp that is homologous (70.45 % identity) to the gene *Mi-1.2*. After *Meloidogyne incognita* inoculation, real-time qPCR showed that the *CaRKNR* expression level was increased from 0.63 to 2.16 times. Using the virus-induced gene silencing system, the *CaRKNR* gene's expression level was reduced significantly than controls, and the average numbers of galls and egg masses in silenced seedlings were 44.39 and 42.01, respectively, while the controls were 0.13. This study revealed that *CaRKNR* was induced by *M. incognita* and its expression correlated with pepper resistance against root-knot nematodes.

Keywords *Capsicum annuum* L. · *Meloidogyne incognita* · Resistance gene · Cloning · Function analysis

Abbreviations

ARC	APAF-1, R proteins, and CED-4
Blast	Basic local alignment search tool
LRR	Leucine-rich repeat
NB	Nucleotide binding
NJ	Neighbour-joining
ORF	Open reading frame
Pfam	Protein family
RACE	Rapid amplification of cDNA end
RKN	Root-knot nematode (*Meloidogyne* spp.)
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SSH	Suppression subtractive hybridization
VIGS	Virus induced gene silencing

Introduction

Root-knot nematodes (*Meloidogyne* spp., RKNs) are root obligate endoparasites that infect numerous plant species and cause serious damage annually to agricultural crops (Abad et al. 2008). RKN second-stage

Electronic supplementary material The online version of this article (doi:10.1007/s10681-015-1438-8) contains supplementary material, which is available to authorized users.

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juveniles (J2) infect roots, move to the root apex, and, upon reaching the zone of cell elongation and the developing vascular cylinder, ultimately become sedentary within the root. At this point, one or more plant cells situated around the head of the J2 are stimulated by repeated stylet probing to undergo mitosis without cytokinesis and differentiate into multi-nucleated giant cells. The giant cells act as sinks, diverting plant nutrients to provide metabolic energy for the nematode. In addition, the hyperplasia and hypertrophy of the surrounding cells lead to the typical root gall (Tytgat et al. 2000).

Breeding of disease resistance is the most efficient strategy for RKN management, especially in Solanaceous plants. Resistance genes are key to the plant's defense against RKNs. The *Mi-1.2* (*Solanum lycopersicum*) is a single dominant gene in tomato that has been cloned. It encodes a protein with a nucleotide-binding site and leucine-rich repeat motif (NBS-LRR) (Milligan et al. 1998), and confers resistance against three major RKNs, *Meloidogyne arenaria*, *Meloidogyne javanica*, and *Meloidogyne incognita* (Gilbert and McGuire 1956). Currently, RKN resistance in commercially available tomato cultivars is conferred only by the *Mi* gene (Huang et al. 2004).

In pepper, some RKN-resistance genes (R genes) have been discovered, but few have been cloned. Two nematode-resistant pepper cultivars (Carolina Wonder and Charleston Belle) have been reported to have the nematode-resistance gene *N* (Thies and Fery 1998). Using doubled-haploid pepper lines, six *Me* genes were found, which clustered on the P9 chromosome (Djian-Caporalino et al. 2007). HDA149 is a nematode-resistant pepper doubled-haploid line that carries the single dominant gene *Me3*, conferring strong RKN resistance (Djian-Caporalino et al. 2001) against three major RKNs, *M. arenaria*, *M. javanica*, and *M. incognita* (Djian-Caporalino et al. 2001), as well as the typical hypersensitive response to RKN infection (Bleve-Zacheo et al. 1998). In this research, we isolated a novel *R* gene, which was induced by RKN and involved in pepper (*Capsicum annuum* L.) resistance against *M. incognita*. These findings could be conducive to breeding new nematode-resistant pepper cultivars.

Using suppression subtractive hybridization (SSH) technology, a SSH cDNA library of pepper root responses to nematode infection was constructed, and a nucleotide-binding site and leucine-rich repeat (NBS-LRR) family gene, *CaRKNR*, was isolated. Based on the isolated gene fragment, the full-length sequence of *CaRKNR* was cloned, and the expression characteristics and functions were analyzed using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and virus-induced gene silencing (VIGS). These results will be useful in revealing the pepper nematode-resistant response, and in pepper breeding for RKN control.

Materials and methods

Biological materials

Pepper line HDA149 was obtained from INRA (Montfavet, France). *Meloidogyne incognita* was grown in a greenhouse at the Chinese Academy of Agricultural Science (Beijing, China). J2 juveniles freshly hatched from egg masses were used as inocula. The pepper HDA149 seedlings were grown in the greenhouse. After 21 days of sowing, single plants were transplanted into 10 × 15 cm (diameter/high) plastic pots that contained a pasteurized mix of sandy loam soil and fine washed river sand (2:1 by volume). The pots were placed in growth chambers programmed to maintain a temperature of 22 °C and 16/8-h (light/dark) cycles (Thies and Fery 1998). 10 days after transplant, each seedling was inoculated with 600 J2 of *M. incognita*. The controls were mock-inoculated with water. Plant roots were harvested 12, 24 and 36 h after inoculation. At each time point, 10 inoculated and 10 mock-inoculated plants were harvested, with three independent biological replicates. Roots were washed, gently dried and sectioned 1–2 cm above the growth tip, snap-frozen in liquid nitrogen, and stored at −80 °C for future use.

Construction of the SSH cDNA library

Total RNA was isolated according to the manufacturer's instructions using Trizol (Invitrogen, Paisley, UK). Poly(A)$^+$ RNAs were purified from total RNA using a mRNA isolation system (Promega, USA). The cDNAs were reverse-transcribed from ~2 μg purified mRNA of each sample. Double-stranded cDNAs were synthesized and amplified using the BD SMART PCR cDNA Synthesis Kit (Takara, Japan). A cDNA sample from root tips inoculated with RKN was used as a

tester, while another sample from root tips mock-inoculated with water was used as a driver; both of these were used to constructed a SSH cDNA library. The cDNA subtractive hybridization and selective amplification of cDNA fragments were performed according to the user manual for the PCR-Select cDNA Subtractive Kit (Takara, Japan). The cDNA fragments specific to the tester were then amplified by a primary PCR consisting of 26 cycles with PCR primer 1 and a secondary PCR of 11 cycles using nested primers 1 and 2R (Table supplement 1, S1). The amplified cDNA fragments, purified with the QIA-quick PCR Purification Kit (Qiagen, USA), were ligated into the pGEM T-easy vector (Promega, USA). Subsequently, the products were introduced into *Escherichia coli* strain Top10 (Tiangen, China). The subtracted cDNA library of HDA149 was constructed. Dot blot hybridization was carried out according to the manufacturer's protocol (DIG Nonradioactive Nucleic Acid Labeling and Detection System kit, Roshi, USA). The differential screening of clones was performed according to the recommended protocol.

Microarray hybridization and signal analysis were carried out as described previously (González-Candelas et al. 2010). Only signal values 1.3 times greater than the corresponding background and derived from at least two replicate hybridizations were taken as valid measurements (López-pérez et al. 2014). Using Sanger dideoxy sequencing technology, these positive clones were sequenced by the Chinese National Human Genome Center (Sino Geno Max Co., Ltd, Beijing, China). All of the expressed sequence tags (ESTs) were submitted to the National Center of Biotechnology Information (NCBI) database of expressed sequence tags (dbEST) and published.

Isolation of *CaRKNR* and sequence analysis

The sequence of the full-length cDNA of *CaRKNR* was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE), using the 5′, 3′-RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen,UK). In the 3′-RACE procedure, mRNAs are converted into cDNAs using reverse transcriptase and an oligo-dT adapter primer (AP). Specific cDNA is then amplified by PCR using a gene-specific primer (*GSP3*) that anneals to a region of known exon sequences and an AP that targets the poly(A) tail region (3′-AUAP). 5′-RACE uses an antisense gene-specific primer (*GSP2*) for the synthesis of specific cDNA by reverse transcriptase. Prior to PCR, a dT-tailing step attaches an adapter sequence to the unknown 5′ sequences of the cDNA. Specific cDNA is then amplified by PCR using a nest GSP (*GSP1*) that anneals in a region of known exon sequences and an AP that targets the 5′ terminus (5′-AUAP). The anchor and APs were selected based on the manufacturer's recommendations.

The entire nucleotide sequences of *CaRKNR* clones were determined using an automatic DNA sequencer (ABI Prism, USA). With the complete ORF primers, *CaRKNR-F* and *CaRKNR-R*, the full-length sequence of *CaRKNR* cDNA was amplified. The *GSP1-3*, *CaRKNR-F*, and *CaRKNR-R* primer sequences are supplied in Table S1.

A phylogenetic analysis, including distance, parsimony, and bootstrap analyses, was performed using Mega (version 6.01). Neighbor-joining (NJ) was the primary method to infer phylogenetic relationships between the NBS genes (Meyers et al. 2003). Numbers on branches indicate the percentage of 1000 bootstrap replicates that support the adjacent node.

RT-qPCR

RT-qPCR was performed to determine the expression pattern of *CaRKNR* in resistant pepper line HDA149. Total RNA samples from root tips, stems, leaves, buds, and young fruits of HDA149 were reverse-transcribed using an oligo-dT nucleotide and the Super Script III Reverse Transcriptase (Invitrogen,UK). qPCR was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) with 1.1 software. The amplification program was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s, using qPCR SYBR Premix Ex Taq II for the fluorophore SYBR green with fluorescein (Takara, Japan). Standard curves were established with five serial dilutions of first-strand cDNA, ranging from 1 to 1/10,000. As a reference, the β-actin cDNA was amplified using the primers *β*-actin F1 and R2 (Table S1). The *CaRKNR* gene-specific primers used for qPCR are provided in Table S1. The relative abundance of transcripts was calculated by the comparative threshold cycle (CT) method (Applied Bio-systems, USA). RT-qPCR was carried out in triplicate for each sample.

Pathogen inoculations

To reveal the *CaRKNR* expression-level changes in pepper roots, 6-week-old pepper plants were challenged by different pathogens, RKN (*M. incognita*), tobacco mosaic virus (TMV), *Ralstonia solanacearum*, and *Phytophthora capsici* Leonian. These plant pathogens were stored in the disease lab of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Science.

Meloidogyne incognita (1000 J2) were inoculated into the rhizosphere soil of each pepper seedling. The TMV inoculum, 1 mg TMV-infected tobacco leaves, were suspended in 5 ml phosphate buffer (50 mM) and then applied to the surface of fully expanded pepper plant leaves and rubbed with Carborundum. *Ralstonia solanacearum* was cultured in triphenyl tetrazolium chloride (TTC) liquid medium at 28 °C. The inocula were collected by centrifuge, re-suspended in sterilized water to 10^6 CFU ml^{-1}, and then infiltrated into the leaves by needleless syringe. *Phytophthora capsici* was cultured on V8 agar medium for 5 days at 25 °C, the cultures were flooded with sterilized water, incubated at 25 °C for 45 min, refrigerated at 5 °C for 15 min, and then kept at room temperature. The suspension was passed through miracloth to separate the fungal material from pieces of agar, homogenized in a low-speed Warren blender for 5 min, and filtered through a double-layer cheesecloth to collect *P. capsici* zoospores (10^5 spores ml^{-1}); these zoospores were used as inocula to spray on the leaves. The pathogens inoculation test included three independent replicates. These inoculated plants were incubated under 100 % relative humidity (Zheng et al. 2011). Plants mock-inoculated with sterile distilled water were controls. These pots were placed in growth chambers programmed to maintain a temperature of 22 °C, with a 16/8-h (light/dark) cycle (Thies and Fery 1998). At 36 h post-inoculation, sections of root tips (1–2 cm) were taken as samples for *CaRKNR* expression-level analysis, and total RNA isolation, DNAase treatment, cDNA synthesis, and RT-qPCR were performed as described above.

VIGS for *CaRKNR*

Using PCR (primers in Table S1), restriction enzymes and ligase, the *CaRKNR* cDNA fragments *CaRKNR-1* (657 bp) and *CaRKNR-2* (416 bp) were cloned into the *Bam*HI-*Kpn*I site of the pTV00 vector. pTV-CaRKNR1, pTV-CaRKNR2, and pTV00 (empty vector) were transformed into *Agrobacterium tumefaciens* strain GV3101, and the transformed cells were selected on YEB media containing 50 mg L^{-1} kanamycin, 15 mg L^{-1} tetracycline, and 50 mg L^{-1} rifampicin. These transformants were grown at 28 °C, centrifuged, re-suspended to an OD600 = 0.5 in buffer (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone), and shaken at 22–25 °C for 5 h. Transformant cultures were mixed at a 1:1 ratio with *A. tumefaciens* containing the tobacco rattle virus vector (TRV1, pBINRA6, OD600 = 0.5) (Liu et al. 2002).

The mixture was infiltrated into cotyledons of 6-week-old HDA149 pepper seedlings using a 5-ml needleless syringe. At the same time, mock inoculations using distilled water were performed as controls. The inoculated plants were transferred to a growth chamber maintained at 16 °C for 1 day with 60 % relative humidity, and then placed in a growth room at 25 °C with a light intensity of ~400 μmol m^{-2} s^{-1} in a 16/8-h light/dark cycle. For each experiment treatment, 10 chili pepper plants were inoculated and with three biological repeats. Ten days after infiltration, 90 positive silenced plants were selected by PCR (primers are listed in Table S1), and inoculated with 3000 J2 *M. incognita* per plant. Of these, 45 plants were used to measure the numbers of root knots and egg masses 8 weeks after nematode inoculation, and the other 45 were used for real-time qPCR analysis of the *CaRKNR* expression level. The VIGS test for *CaRKNR* was performed with three repeats. The numbers of galls and egg masses were analyzed using Duncan's new multiple range method, available in the ANOVA section of the SAS9.1 software.

Results and analysis

Full length of *CaRKNR* cDNA

Using SSH, a smeared PCR product was produced, ranging largely from 200 to 700 bp. Differential screening resulted in 1200 positive clones from the library. Based on the blot and sequencing, 211 ESTs were identified as either submergence-induced or highly expressed after differential screening of the HDA149 SSH cDNA library. These EST sequences were published on the NCBI dbEST database (JZ820466-JZ820676). The EST H-634 (JZ820592)

was homologous to the nematode-resistance gene *Mi-1* copy 2 (U65668.1) (Fig. S1), with 100 % coverage and 85 % identity. Thus, H-634 was confirmed as a candidate pepper nematode-resistance gene and named *CaRKNR*.

Based on the 610-bp nucleotide sequence of the *CaRKNR* gene fragment, the full-length cDNA sequence was obtained using RACE technology (GenBank accession number FJ231739). The full-length cDNA of *CaRKNR* is 4697 bp long, and includes a putative transcription start site, a potential open reading frame of 3600 bp, 123 bp 5′- and 974 bp 3′-untranslated regions, and a poly(A) tail. The potential open reading frame encodes 1199 amino acid residues (ACI43068.1).

Sequence alignment and phylogenetic analysis

The protein family (Pfam) blast (http://pfam.sanger.ac.uk/search) identified two Pfam-A matches to the CaRKNR sequence, a NB-ARC domain (467–747) and a LRR domain (894–954), which belonged to the gene families NB-ARC (PF00931) and LRR 8 (PF13855), respectively.

The CaRKNR protein sequence was blasted in NCBI (www.ncbi.nlm.nih.gov) using the BlastP program, and the first 38 proteins with maximum E values of 0, and 61 proteins with E values more than 2e-101, belonged to the AAA superfamily, which included a conserved NB-ARC domain. From these proteins, 23 were selected to construct a phylogenetic tree, which showed that CaRKNR and the disease resistance homolog Mi1-2 (AAC32252.1) in tomato (*S. lycopersicum*) were clustered together (Fig. 1). The homolog analysis (DNAman 6.0) showed that the protein sequence of CaRKNR was 70.45 %, identical to that of the RKN-resistance protein Mi-1.2. The motifs of 10 homologous genes were analyzed by MEME (version 4.9.1, http://meme.nbcr.net/), which identified three common motifs among these genes. The locations of motifs 1 and 2 overlapped with the conserve domains NB-ARC and LRR (Figs. 2, S2). This revealed that the *CaRKNR* gene belonged to the NBS-LRR gene family.

Expression pattern analysis of *CaRKNR*

RT-qPCR was used to analyze *CaRKNR* expression levels in different tissues of resistant pepper line HDA149. The results indicated that *CaRKNR* was highly expressed in the bud, ~4.9 times greater than the reference gene β-actin, was weakly expressed in fruit, stem and root (0.2, 0.5 and 0.6 times the reference gene, respectively), and was undetectable in the leaves (Fig. 3).

The pepper HDA149 was challenged by different pathogens, RKN (*M. incognita*), TMV, *R. solanacearum*, and *P. capsici* Leonian. The RT-qPCR revealed that the *CaRKNR* transcript level of the control was 0.467 time, while that of the RKN treatment was 1.523, 3.26 times higher than the control. The *CaRKNR* transcript levels after TMV, *R. solanacearum*, and *P. capsici* treatments were 0.492, 0.467 and 0.375 time, respectively (Fig. 4), and Duncan's new multiple range method showed that the ANOVA sum of squares and mean square were 2.861 and 0.715, respectively ($F = 91.65$, P value < 0.0001). The *CaRKNR* transcript level in RKN plants was significantly higher than in plants receiving other treatments, but the differences among the control, TMV, *R. solanacearum*, and *P. capsici* treatments were not significant, indicating that the *CaRKNR* gene responded to RKN.

VIGS of *CaRKNR*

8 weeks after nematode inoculation, *CaRKNR* VIGS effects were detected (Fig. 5). In the controls with mock Agrobacterium buffer and the pTV00 empty vector (negative control), the gall numbers were only 0.13 and 0.18, respectively. However, for the pTV-CaRKNR1 and pTV-CaRKNR2 treatments, the average numbers of galls were 50.22 and 38.56, respectively (Fig. 6). Compared with the control, the galls of *CaRKNR*-silenced seedlings were significantly increased ($F = 71.31$ and $P < 0.0001$). In addition, there was also a significant difference between the VIGS pTV-CaRKNR1 and pTV-CaRKNR2 treatments. The statistics of egg masses for VIGS treatments had a similar results with galls ($F = 118.62$ and $P < 0.001$). These showed that different cDNA fragments caused different VIGS effects. The VIGS effects revealed that *CaRKNR* has a RKN-resistance function.

The effect of VIGS was analyzed at the RNA level by real-time RT-qPCR. After agro-infiltration, the normalized *CaRKNR* mRNA levels of the pTV00 and control plants were 0.63 and 0.57 times, respectively, but for the pTV-CaRKNR1 and pTV-CaRKNR2 treatments the levels were 0.27 and 0.41 times, respectively, reflecting decreases of 57.1 and 34.7 %

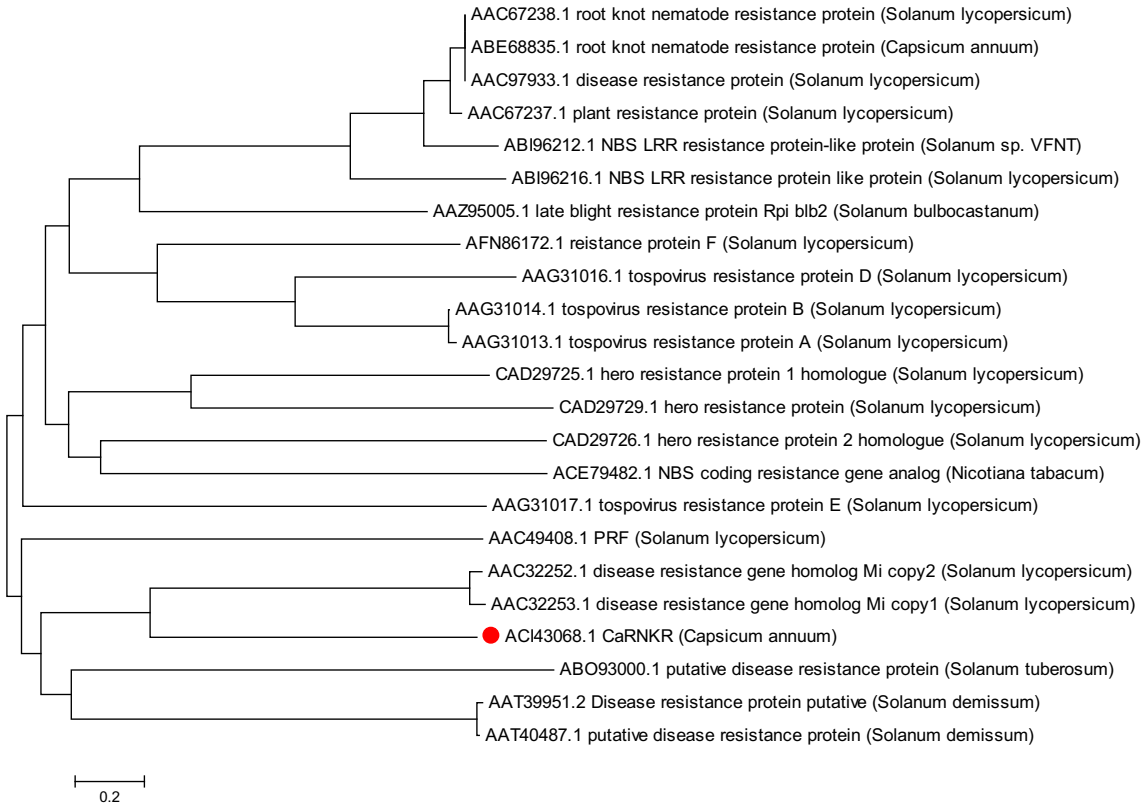

Fig. 1 Phylogenetic tree analysis of a nucleotide-binding site and leucine-rich repeat (NBS-LRR) family protein from pepper (*Capsicum annuum* L.), CaRKNR, and some resistance proteins. A total of 23 proteins encoded by resistance genes were analyzed in this phylogenetic tree. The *red dot* indicates CaRKNR (ACI43068), and the other protein sequences were downloaded from NCBI (www.ncbi.nlm.nih.gov). *Solanum lycopersicum* sequences: AAC97933.1, AAC32252.1, AAC67238.1, AAC67237.1, AAC32253.1, ABI96216.1, CAD29725.1, CAD29726.1, CAD29729.1, AAG31014.1, AAG31013.1, AAG31017.1, AFN86172.1, AAG31016.1 and AAC49408.1; *Capsicum annuum,* ABE68835.1; *Solanum bulbocastanum*, AAZ95005.1; *Solanum* sp. VFNT, ABI96212.1; *Solanum demissum*, AAT39951.2; *Solanum tuberosum*, ABO93000.1; *Nicotiana tabacum*, ACE79482.1; and *Solanum demissum*, AAT40487.1. The *bar* shows 0.2 scale length

compared with the controls. After RKN inoculation from 0 to 7 days, the *CaRKNR* mRNA level of control plants was increased significantly from 0.63 to 2.16. However, in silencing plants, the *CaRKNR* mRNA level showed only a slow increase (Fig. 7). These RT-qPCR results revealed that the *CaRKNR* mRNA in VIGS plants was silenced effectively.

Discussion

CaRKNR is a novel resistance gene belonging to the NBS-LRR family

Using SSH technology, *CaRKNR* was isolated and cloned from the nematode-resistant pepper HDA149. Its encoded protein has a domain structure, including a central conserved region with NBS and C-terminal LRR domains, which are characteristics of the NBS-LRR family of resistance genes (Belkhadir et al. 2004). NBS-LRR motifs are common in nematode R genes, such as *Mi-1*, *Hero A*, *Gpa2*, *Gro1*, and *Ma* (Milligan et al. 1998; Ernst et al. 2002; Van der Vossen et al. 2000; Paal et al. 2004; Claverie et al. 2004; Chen et al. 2007). The CaRKNR protein was homologous to the *Mi-1.2* protein, and 70.45 % of the sequence was identical. *Mi-1.2* from tomato is a member of the NBS-LRR family that has been cloned and is located on chromosome 6 (Ammiraju et al. 2003). It offers resistance to the RKNs *M. javanica*, *M. incognita*, and *M. arenaria*, and triggers a localized tissue necrosis or hypersensitive response (Milligan et al. 1998). At

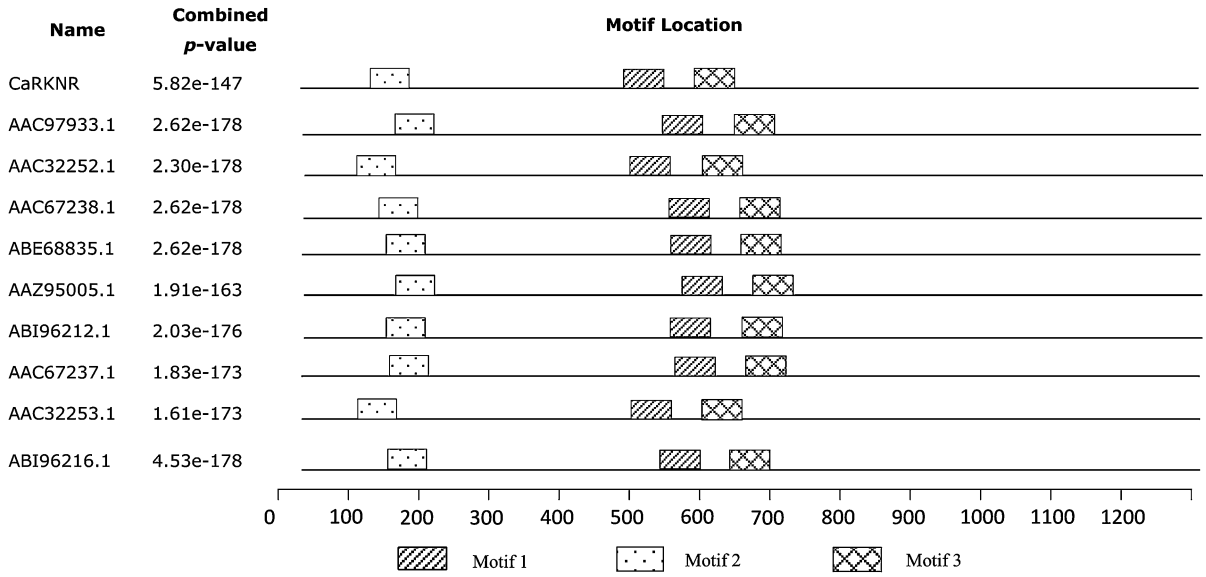

Fig. 2 Analysis of three conserved motifs among the homologous proteins identified by MEME. *Rectangles* shaded with *diagonal lines*, *spots*, and *grids* indicate the locations of motifs 1–3, respectively. The *line* represents the sequences of homologous proteins and the *ruler* shows the lengths of the sequences. Protein sequences AAC97933.1, AAC32252.1, AAC67238.1, AAC67237.1, AAC32253.1, and ABI96216.1 are from *Solanum lycopersicum*, CaRKNR (ACI43068) and ABE68835.1 are from *Capsicum annuum*, AAZ95005.1 is from *Solanum bulbocastanum*, and ABI96212.1 is from *Solanum* sp. VFNT

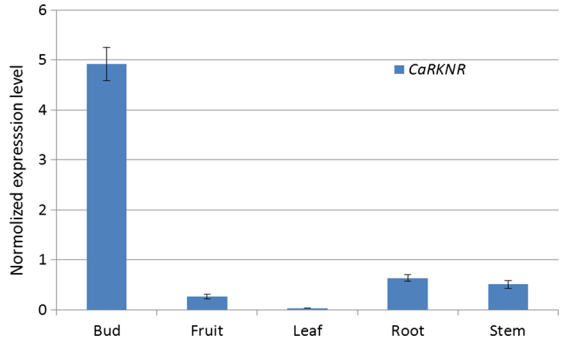

Fig. 3 RT-qPCR analysis of the *CaRKNR* expression level in different tissues of pepper HDA149. In this diagram, five *bar graphs* indicate the *CaRKNR* expression levels in bud, fruit, leaf, root, and stem samples of the pepper HDA149. Each *bar graph* represents the mean ± SD of three independent biological replicates

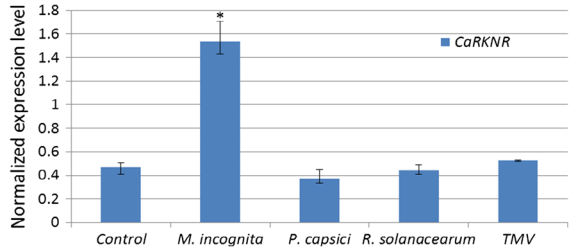

Fig. 4 qPCR analysis of the *CaRKNR* expression level in pepper HDA149 challenged by four pathogens. Five *bar graphs* indicate the *CaRKNR* expression levels in pepper HDA149 root tissues and in the same tissue after the pepper plants were challenged by the pathogens *Meloidogyne incognita*, tobacco mosaic virus (TMV), *Ralstonia solanacearum*, and *Phytophthora capsici* Leonian. Each *bar graph* represents the mean ± SD of three independent biological replicates. The *asterisk* indicates significant difference at the $P = 0.05$ level by an ANOVA statistical analysis, the F value = 91.65, P value < 0.0001

present, nine genes of the *Mi* family have been reported in tomato (Jablonska et al. 2007). *Mi-1* was the first RKN-resistance gene to be cloned (Milligan et al. 1998), and was used exclusively for 60 years in breeding tomatoes resistant to RKN. Thus, finding and exploiting novel nematode-resistant genes is important to control RKNs.

In pepper, six *Me* genes have been reported, and they cluster in a single genomic region within a 28-cM interval on chromosome P9. This genomic area is colinear to chromosome T12 of tomato and chromosome XII of potato (Djian-Caporalino et al. 2007). Not all of the *Me* genes have been cloned, but it was reported

Fig. 5 Root galls and egg masses on pepper HDA149 seedlings after virus-induced gene silencing (VIGS) treatments. The numbers of galls and egg masses on the VIGS-treated seedlings (part **a**, pTV-CaRKNR1 and part **b**, pTV-CaRKNR2) were significantly greater than on the negative control (part **c**, empty pTV00) and mock (part **d**, buffer). The *arrows* indicate the gall and egg masses. CaRKNR: a nucleotide-binding site and leucine-rich repeat (NBS-LRR) family gene fragment from pepper (*Capsicum annuum* L.)

that HDA149 had a single dominant nematode resistance gene *Me3* that induced localized cell necrosis in infected plants (Bleve-Zacheo et al. 1998). *CaRKNR* was cloned from HDA149, but it was necessary to determine whether it was a homolog of *Me3* or another *Me* gene in pepper. Recently, the genome sequences of pepper were published, providing an important basis for *CaRKNR* gene mapping to determine the relationships among the pepper's nematode-resistant genes (Kim et al. 2014; Qin et al. 2014). With the program Blast, *CaRKNR* was searched in the pepper Zunla1 (*Capsicum annuum* L.) genome database (accession ASJU0 0000000, release 2.0) (http://peppersequence.genomics.cn), results showed that *CaRKNR* was mapped on the

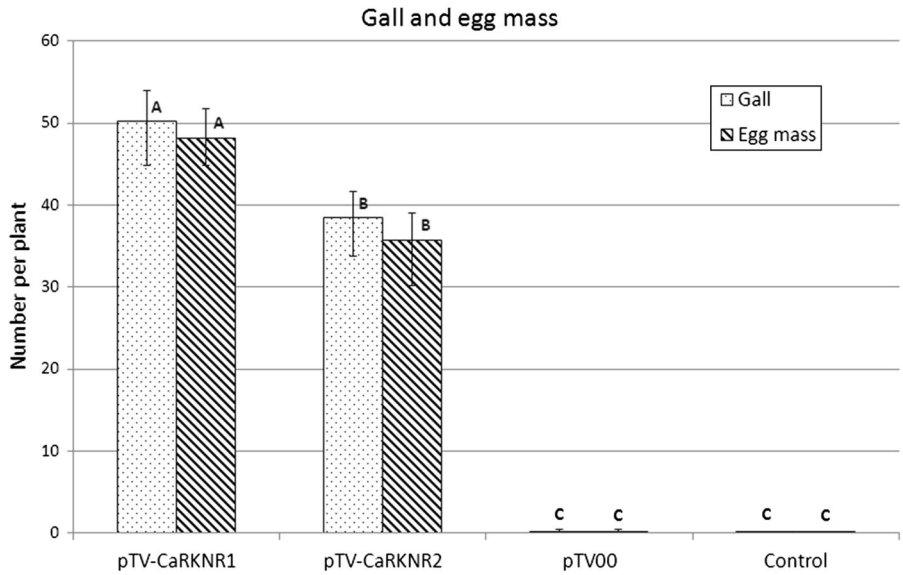

Fig. 6 Gall and egg mass statistical analysis for different treatments in the *CaRKNR* VIGS test. *Bar graphs* indicate the numbers of galls and egg masses after different VIGS treatments, pTV-CaRNR1, pTV-CaRNR2, pTV00 and a mock control. The *bar graphs* shaded with *spots* indicate the gall quantity, and the *bar graphs* shaded with *diagonals* indicate the egg mass quantity. Each *bar graph* represents the mean ± SD of three independent biological replicates. The *capital letters* (A, B, C) on each *bar graph* indicate significant difference at $P = 0.01$ by ANOVA statistical analysis (Gall F value = 1097.70, $P < 0.0001$; Egg mass F value = 905.14, $P < 0.0001$), and the *same letters* are not significantly *different*

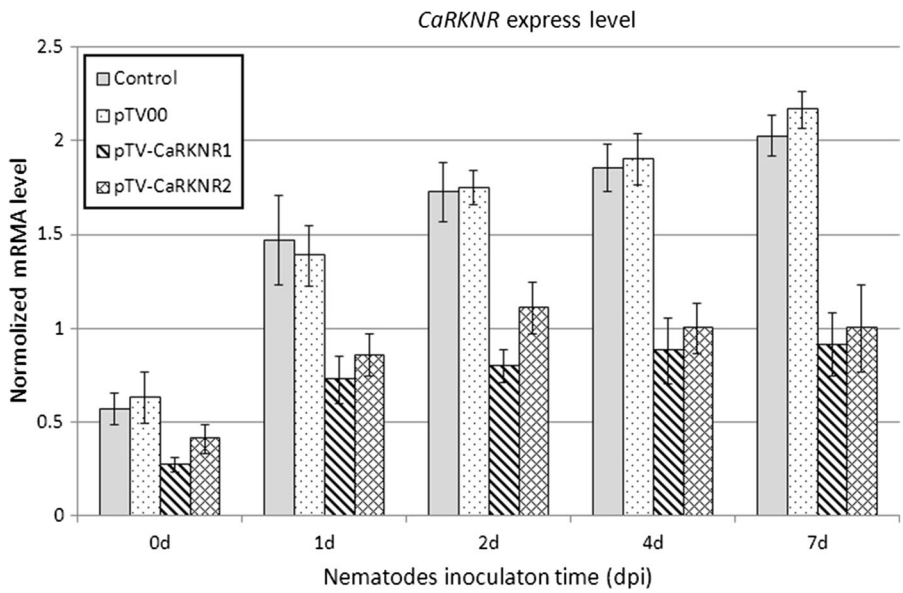

Fig. 7 Pepper HDA149 *CaRKNR* mRNA level analysis in different VIGS treatments. The *bar graphs* in the diagram indicate the *CaRKNR* expression levels from 0 to 7 days after RKN inoculation; *bars* shaded with *gray*, *spots*, *diagonal lines*, and a *grid* indicate the control, pTV00, pTV-CaRKNR1 and pTV-CaRKNR2 treatments, respectively

chromosome 6, the Expect = 0.0, Identities = 99.5 %, covered from 219,292,195 to 219,295,794 bp of chromosome 6. Similar, in the Pepper CM334 (*Capsicum annuum* cv.) genome (AYRZ00000000) CDS database (V1.55) and chromosome database (V1.55), *CaRKNR* was also mapped on the chromosome 6, the alignment

gene was CA00g56860, and Expect = 0.0, Identities = 99.7 %. From these results we putative that *CaRKNR* is on chromosome 6, not 9, don't belong to *Me* family genes, but a novel NBS-LRR gene member from pepper.

CaRKNR is involved in *M. incognita* resistance

VIGS is an effective technology for the functional characterization analysis of genes involved in nematode resistance. *Mi* silencing correlated with the resistance-breaking phenotype and successful nematode development (Valentine et al. 2004). The study of a TRV-*Mi* construct indicated that the heat-stable resistance gene *Mi-9* is mediated by a homolog of *Mi-1* (Jablonska et al. 2007). Using VIGS, we found that the gene silencing of *CaRKNR* resulted in increased production of galls and egg masses in the pepper HDA149. The average number of galls after the two VIGS treatments (pTV-CaRKNR1 and pTV-CaRKNR2) was 44.39, and the egg mass was 42.01, which was significantly more than the empty vector control (0.13) or mock control (0.18), indicating that the resistance of pepper HDA149 against nematodes was weakened. At the same time, real-time RT-qPCR showed that the normalized mRNA level of *CaRKNR* was decreased in the silenced plants. At 7 days post-RKN inoculation, the average level was 0.95 time, but the control was 2.16 times, indicating that the VIGS treatments were effective in decreasing the *CaRKNR* expression level. The results of the VIGS tests and qPCR analysis demonstrated that *CaRKNR* functions are involved in the pepper's *M. incognita* resistance.

The expression characteristics analysis showed that the *CaRKNR* gene responds to RKN and that after the inoculation of nematodes, the expression level was significantly increased. However, the qPCR data indicated that *CaRKNR* was more highly expressed in the bud tissue than in the root tissue, which is interesting. Since the bud tissue undergoes active growth and metabolism, the high *CaRKNR* expression level could be related to this activity. *Mi-1* conferred resistance to RKNs, and the *Mi* gene was also involved in resistance to some potato aphids (*Macrosiphum euphorbiae*) and the sweet potato whitefly (*Bemisia tabaci*) (Rossi et al. 1998; Nombela et al. 2003). Whether the *CaRKNR* gene's high expression level in buds has a resistance function remains to be determined.

Conclusion

CaRKNR, a novel NBS-LRR family gene, was cloned from the nematode-resistant pepper HDA149. A RT-qPCR analysis showed that the expression of *CaRKNR* was induced by *M. incognita* in pepper roots. The VIGS tests indicated that *CaRKNR* expression levels were decreased effectively in root, and that the numbers of galls and egg masses were significantly increased compared with the control. Thus, the pepper resistance to nematodes was weakened. This study revealed that *CaRKNR* was involved in pepper resistance against *M. incognita*, which may be important in pepper breeding.

Acknowledgments We thank INRA Versailles for providing the pepper HDA149. This work was supported by the National Science Foundation of China (30971905, 31101425), Agro-scientific Research in the Public Interest (201103018) and the China Agriculture Research System (CARS-25).

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