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CELL BIOLOGY AND MORPHOGENESIS

# *CaMi*, a root-knot nematode resistance gene from hot pepper (*Capsium annuum* L.) confers nematode resistance in tomato

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**Abstract** Several root-knot nematode (*Meloidogyne* spp.) resistance genes have been discovered in different pepper (*Capsium annuum* L.) lines; however, none of them has yet been cloned. In this study, a candidate root-knot nematode resistance gene (designated as *CaMi*) was isolated from the resistant pepper line PR 205 by degenerate PCR amplification combined with the RACE technique. Expression profiling analysis revealed that this gene was highly expressed in roots, leaves, and flowers and expressed at a lower level in stems and was not detectable in fruits. To verify the function of *CaMi*, a sense vector containing the genomic DNA spanning the full coding region of *CaMi* was constructed and transferred into root-knot nematode susceptible tomato plants. Sixteen transgenic plants carrying one to five copies of T-DNA inserts were generated from two nematode susceptible tomato cultivars. RT-PCR analysis revealed that the expression levels of *CaMi* gene varied in different transgenic plants. Nematode assays showed that the resistance to root-knot nematodes was significantly improved in some transgenic lines compared to untransformed susceptible plants, and that the resistance was inheritable. Ultrastructure analysis showed that nematodes led to the formation of galls or root knots in the susceptible lines while in the resistant transgenic plants, the *CaMi* gene triggered a hypersensitive response (HR) as well as many necrotic cells around nematodes.

**Keywords** *CaMi* · Gene cloning · Root-knot nematode · Pepper

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## Introduction

The root-knot nematodes *(Meloidogyne* spp.) are the major pepper pest throughout the world (Di Vito et al. 1985; Thomas et al. 1995). These parasites are prevalent in open fields and controlled environment where several nematode generations can be completed within 1 year (Djian-Caporalino et al. 1999). The infective juveniles move intercellularly after penetrating the roots, migrating down the plant cortex towards the tip. They then enter the base of the vascular cylinder and migrate up the root (Wyss et al. 1992) and establish a permanent feeding site in the differentiation zone of the roots by inducing nuclear division without cytokinesis in host cells (Williamson and Gleason 2003). This process gives rise to large, multinucleate cells, termed giant cells, which cause the formation of galls or root knots (Williamson and Hussey 1996). These alterations severely affect the uptake of water and nutrients and interfere with the translocation of minerals and pho-

tosynthates in the host (Milligan et al. 1998), resulting in wilted and stunted plants with significantly reduced yield. Moreover, it makes the host plants more susceptible to other soil-borne pathogens, usually bacteria and fungi (Castaagnone-Sereno et al. 1992).

Currently, the primary method to control nematodes is soil fumigation, and the principal fumigant used is methyl bromide (Fery and Dukes 1996). However, environmental concerns and government regulations promote the use of non-chemical over chemical pest control methods. Cultivars resistant to this pest would potentially render soil fumigant and toxic systemic nematicides unnecessary as they would be an efficient and durable control method (Djian-Caporalino et al. 1999).

Resistance to diverse pathogens, including viruses, bacteria, fungi and nematodes, has been shown to be genetically mediated by a single dominant resistance gene (*R* gene) in the host. Each of these *R* gene products interacts directly or indirectly with the product of a corresponding avirulence (*Avr*) gene in the pathogen (Flor 1971; Keen 1990). To date, over 30 *R* genes have been cloned from several different plant species (Milligan et al. 1998; Ferrier-Cana et al. 2003). Based on the deduced secondary structure of their protein products, *R* genes can be divided into five broad classes (Dangl and Jones 2001). The largest *R* gene class encodes proteins with a nucleotide binding site (NBS) domain followed by a leucine rich repeat (LRR) region at the C terminus. Several conserved motifs have been identified in the NBS–LRR domains. The NBS domain contains three peptide motifs (kinase-1a (P-loop), kinase 2 and kinase 3a) that are critical for nucleotide binding in many ATP/GTP binding proteins (Meyers et al. 1999). The C-terminal LRR domain is shared by many other proteins and functions as a region for protein–protein interactions and peptide–ligand binding. The LRR domain of R proteins might contribute to the recognition of diverse pathogen-derived ligands (Tameling et al. 2002).

Resistance to root-knot nematode was first identified 60 years ago in an accession (PI128657) of *Lycopersicum peruvianum* Mill, a wild relative of cultivated tomato (*L. esculentum* Mill.) and originated in the western coastal region of South America (Watts 1947). The single dominant *Mi* gene of tomato confers resistance to three major root-knot nematodes (Gilbert and McGuire 1956) and has been isolated by positional cloning approach (Milligan et al. 1998). It shares several structural motifs with other *R* genes, including NBS and LRR domains, which are characteristic of a family of plant proteins that are required for resistance against viruses, bacteria, fungi and nematodes. The *Mi* locus contains three open reading frames. Two of them, *Mi-1.1* and *Mi-1.2*, appear to be intact genes; while the third is a pseudogene. Complementation studies revealed that *Mi-1.2*, not *Mi-1.1*, was sufficient in conferring resistance to *M. javanica*. When nematodes became attracted to and penetrated the roots, the *Mi* gene triggered a localized tissue necrosis or hypersensitive response (HR). Nematodes failed in such cases to establish feeding sites and then either died or left the roots. In *Capsicum*, several root knot nematode resistance genes have been discovered (Lindsey and Clayshulte 1982; Fery and Dukes 1984; Thies et al. 1997). As early as 1956, Hare identified a dominant gene (named *N*) which confers resistance to *M. incognita* in the *C. frutescens* L. ''Santanka XS'' line. More recently, Di Vito and Saccardo (1979) and Di Vito et al. (1992) discovered high levels of resistance to root knot nematode in some lines of *C. chacoense* Hunz., *C. chinense* Jacq. and *C. frutescens*. Hendy et al. (1983) found that two *C. annuum* lines, PM217 and PM687, were resistant to a wide variety of root knot nematode populations.

Although a number of root-knot nematode resistance genes (*R* genes) have been discovered in pepper, none of them has been cloned. The isolation of *R* genes has historically involved map-based cloning or transposon tagging, both of which are extremely labor-intensive and expensive strategies. The common features shared by *R* genes have led to new cloning strategies (Rossi et al. 2003). Recently, degenerate primers were designed based on the highly conserved amino acids sequences of known R genes and have been used to successfully amplify multiple DNA sequences from a number of plant species (Deng et al. 2000). These sequences have been called resistance-gene analogs (RGAs) or resistance-gene candidates (RGCs). This PCR-cloning strategy has been employed to clone RGA or RGC sequences from soybean (Penuela et al. 2002), potato (Leister et al. 1996), lettuce (Shen et al. 1998), rice (Leister et al. 1998), Arabidopsis (Aarts et al. 1998), maize (Collins et al. 1998), cassava (Lopez et al. 2003), and other angiosperm species.

In this study, we screened various accessions of hot pepper (*C. annuum*) for nematode resistance and then cloned a root-knot nematode resistance gene (designated as *CaMi*) from the resistant hot pepper accession PR 205. The function of this candidate gene was further verified by transforming the genomic DNA containing the full coding region of *CaMi* in sense orientation into the susceptible tomato cultivars.

## Materials and methods

### Plant materials

Six hot pepper lines, Bianhong No.1, Bianjiao No.1, Sujiao No.5, Chufeng, Xiangjiao No.21, and PR 205, were grown in the greenhouse and used to evaluate their resistance to root-knot nematode. The resistant accession PR 205 was used to isolate the *CaMi* gene and to investigate the expression pattern of *CaMi*.

Three tomato cultivars, Zhongshu 5 (ZS-5), Jia 8 (J-8), and RN-1, were also tested for their root-knot nematode resistance. The susceptible accessions, ZS-5 and J-8, were used for *CaMi* gene transformation.

### Degenerate PCR amplification

Genomic DNA was extracted from young leaves of pepper PR 205 plants using the method described by Fulton et al. (1995). Consensus analysis of NBS and LRR conserved motif of various resistant (R) genes including *Mi, Prf, I2C-1, Hero*, and *RMP1*, revealed that four amino acid regions (Kinase-1a, Kinase-2, Kinase-3a and hydrophobic domain) were highly conserved. Two degenerate primers, DP1 and DP2 (Table 1), were designed from the two conserved regions of Kinase-1a and hydrophobic domain. The PCR amplification reaction (25 μl) contained 1× PCR buffer with 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.5 μM of each primers, 0.2 mM dNTPs, 1.0 U *Taq* polymerase (MBI Fermentas, USA) and 100 ng template DNA. PCR was performed as following: 94°C 5 min; 35 cycles of 94°C 1 min, 55°C 1 min and 72°C 1 min; followed by 72°C for 10 min and held at 4°C using a PTC-100 Thermal Controller (M.L. Research Inc., Waltham, MA, USA). The amplified products were separated by electrophoresis in 0.8% (w/v) agarose gel. The target PCR product was cleaned up by a Gel extraction kit (Promega), cloned into a pMD18-T plasmid vector (TaKaRa, Japan), and transformed into *E. coli* DH5α. Positive clones were confirmed by digestion and sequencing.

### Rapid amplification of cDNA 5′ and 3′ ends (RACE)

To obtain the full-length cDNA of *CaMi*, 5′ and 3′ RACE were performed with the specific primers, SP1 and SP2 (Table 1), using the 'SMART™ RACE cDNA Amplification Kit (Clontech, USA). Three microgram of total RNA from the roots were reverse-transcribed according to the manufacture's instructions. RACE was carried out under the following program: 94°C 3 min; 35 cycles of 94°C 1 min, 56°C 1 min 72°C 4 min; followed by 72°C 10 min. For full-length cDNA amplification, PCR was performed using 5′ and 3′ RACE products for 35 cycles (5 cycles at 94°C for 1 min and 68°C for 6 min; 30 cycles at 94°C for 45 s, 65°C for 1 min, and 72°C for 6 min), followed by an extension step of 10 min at 72°C. The 50 μl PCR reaction mixture contained 1× PCR buffer (MBI Fermentas, Lithuania), 2.0 mM $MgSO_4$, 0.5 μM 10× UPM primers, 0.2 mM dNTPs, 1.0 U *Taq* polymerase, 0.5 U *pfu* polymerase and 100 ng 5′ and 3′ RACE products. The full-length cDNA products were gel purified, cloned into pMD18-T vector, and then sequenced.

Based on the full-length cDNA sequence of *CaMi*, two specific primers, SP3 and SP4, were designed with the enzyme sites *Bam*HI and *Sac*I at the 5′ end, respectively (Table 1). The Ca*Mi* genomic DNA was amplified using these two primers and the resulted genomic PCR fragment was cleaned up by the Gel extraction kit (Promega).

### Construction of the sense *CaMi* vector

The genomic PCR product containing the full coding region of *CaMi* was digested by *Bam*HI and *Sac*I and

**Table 1** Primer sequences used in this study to isolate the *CaMi* gene and identify the transgenic plants

| Name | Sequences |
|---|---|
| DP1 | 5′-TG(G/C)(G/C)(G/A)GG(T/A/C)(T/A)(T/C)(G/A)GG(T/C/G)AAAACTAC-3′ |
| DP2 | 5′-(T/A/C)(G/A)C(T/A)A(A/G)AGG(A/G/C)A(A/G)CCCT(T/C)(T/G/C)ACA-3′ |
| SP1 | 5′-TCAGTCAAGTTAGTGGCTCAGAT-3′ |
| SP2 | 5′-CTCATCCAAGGTAGTAGTATCCC-3′ |
| SP3 | 5′-CGCGGATCCTCCAATAGCTTCAACATTAT-3′ |
| SP4 | 5′-CGCGAGCTCAGAGGAATCTCATCACAGGA-3′ |
| SP5 | 5′-AGATGTCGGTAAAGAAATAGCC-3′ |
| SP6 | 5′-ATCCACGGACAGCACTCG-3′ |
| *β*-actin FW | 5′-ATGGCAGACGGAGAGGATATTCA-3′ |
| *β*-actin RV | 5′-GCCTTTGCAATCCACATCTGCTG-3′ |
| *NPT*II FW | 5′-AGACAATCGGCTGCTCTGAT-3′ |
| *NPT*II RV | 5′-TCATTTCGAACCCCAGAGTC-3′ |

cloned into the binary vector pBI121. The resulting plasmid construct, which we designated as pBCaMi (Fig. 1), was sequenced and transformed into *Agrobacterium tumefaciens* LBA4404 by electroporation.

### Total RNA isolation and RT-PCR analysis

Reverse transcriptase-PCR (RT-PCR) was performed to determine the expression pattern of *CaMi* in resistant pepper line and in transgenic tomato plants. Total RNA was extracted from the roots, stems, leaves, flowers and fruits of resistant pepper line and the young leaves of tomato transgenic plants using Trizol® reagent according to the manufacture's recommended protocol (Invitrogen, USA). Three microgram of total RNA were reverse-transcribed using the RT-PCR HIGH kit (Toyobo, Tokyo, Japan). The resultant first-strand cDNAs were diluted into 100 µl with RNase-free water. A 900 bp cDNA fragment of *CaMi* gene was amplified with the two specific primers, SP5 and SP6 (Table 1), using the following PCR cycles: 94°C 30 s, 56°C 30 s, 72°C 90 s, 24 cycles. The specific primers (Table 1) of *ß*-actin gene, which served as internal standards for RNA quantity, were added to the same RT-PCR reactions.

### Generation and analysis of transgenic plants

Tomato transformation by *Agrobacterium*-mediated was performed as described by Fillatti et al. (1987). Regenerated transgenic plants were screened for *NPT*II gene by PCR amplification. Southern blot was performed to determine the number of T-DNA inserts in each of the transgenic tomato plants. About 15 µg DNA was digested with *Eco*R V (MBI, Fermatas) and separated on 0.8%(w/v) agarose gel in 1× TAE buffer, followed by blotting onto Amershan Hybond-$N^+$ nylon membranes in 0.4 M NaOH. The membranes were hybridized with the [$\alpha^{32}$P]-dCTP labeled probe of the *NPT*II gene fragment at 65°C in a phosphorus buffer (0.5 M) containing 7% SDS, 1% BSA, 1 mM EDTA; and then washed with 2× SSC, 0.5% SDS at room temperature for 10 min and 0.2× SSC, 0.1%(w/v) SDS at 65°C for 5 min. To determine the expression of *CaMi* gene in transgenic plants, RT-PCR was performed as described above.

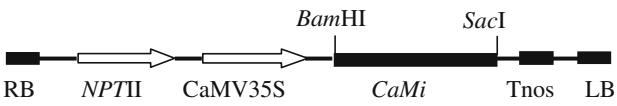


**Fig. 1** Schematic diagram of the T-DNA region of the binary vector pBCaMi. *RB* Right border, *LB* left border, *NPTII* neomycin phosphotransferase gene, *CaMV35S* cauliflower mosaic virus 35S promoter, *Tnos* nopaline synthase terminator, *CaMi* the pepper root-knot nematode resistance gene

### Nematode assay

Second-stage juveniles (J2) of *M. incognita* that had hatched within a 24 h period were collected from a hydroponic culture system (Lambert et al. 1992) or J2 collected from *M. incognita*-infected tomato roots were used as inoculums. Five cuttings of each independent transgenic plant were used for assay of nematode resistance. Transgenic plants, as well as tomato cultivar of ZS-5, line RN-1 and pepper line PR 205 which were served as controls, were inoculated with nematodes according to the procedure described by Yaghoobi et al. (1995). Four to 6-week-old plants were infected with approximately 3,000 J2 of nematodes. Roots were harvested 6 to 8-week later, then carefully washed individually with tap water and stained for 10 min in a solution of erioglaucin (0.05%, Sigerma), a dye shown to specifically stain egg masses blue (Omuega et al. 1988). The roots were then rinsed and examined under a magnifying glass and the number of egg-masses was counted for each tomato plant. Plants were classified as resistance (R) if the individual root system had less than 25 egg masses or susceptible if the individual root system had 25 or more egg masses (Ammiraju et al. 2003).

### Root microscopy

Roots from tomato plants were fixed in formalin-acetic acid-alcohol (FAA) for 24 h and intenerated in hydrofluoric acid for 10–15 days. Subsequently the roots were stained in hematoxalin and then dehydrated with each grade ethanol, re-stained with 1% eosin, infiltrated with paraffin using chloroform as solvent, and gradually embedded with paraffin. Then the specimens were cut into 8–10 µm-thick sections and mounted on microscope slides. To remove paraffin, the slides were immersed in xylene twice for 20 min and enveloped with neutral resin. The finished slides were cured in a 42°C oven until dry. Photomicrographs were made with a Nikon microscope.

## Results

### Screening the accessions of hot pepper (*C. annuum*) and tomato (*L. esculentum*) against root-knot nematodes

Six hot pepper lines, Bianhong No.1, Bianjiao No.1, Sujiao No.5, Chufeng, Xiangjiao No.21, and PR 205,

and three tomato cultivars, Zhongshu 5 (ZS-5), Jia 8 (J-8), and RN-1, were tested for their resistance to root-knot nematodes. After the *M. incognita* infection, only the pepper line PR 205 and the tomato line RN-1 showed resistance while the other lines (Bianhong No.1, Bianjiao No.1, Sujiao No.5, Chufeng, Xiangjiao No.21, ZS-5 and J-8) were all susceptible.

## Isolation of *CaMi* and sequence analysis

The resistant pepper line PR 205 was used to isolate the *CaMi* gene. A 503 bp fragment was obtained through PCR amplification, and based on the nucleotide sequence of this fragment, the full-length cDNA sequence of *CaMi* was obtained using RACE amplification. The full-length cDNA of *CaMi* was 3,986 bp long, which included the putative transcription start site, a potential open reading frame of 3,774 bp, a 5′-untranslated region (5′-UTR) of 86 bp and a 3′-untranslated region (3′-UTR) of 108 bp, including the poly(A) tail. The potential open reading frame encoded for 1,257 amino acid residues and contained a potential leucine zipper and a heptad repeat motif. The predicted nucleotide binding site (NBS) domain was comprised of the kinase-1a (P loop), kinase-2 and kinase-3a motif. At the C terminus, it contained a leucine-rich repeat (LRR) domain (Fig. 2).

Using the specific primers SP3 and SP4 designed based on *CaMi* cDNA sequence information, a genomic fragment with approximately 5.3 kb in length was amplified from the pepper line PR 205. The genomic sequence of *CaMi* was deposited into the GenBank database under the accession number of DQ465824. Comparison between the cDNA and genomic sequences revealed that *CaMi* gene contains two introns near the 5′ end. Intron 1 and intron 2 are 1,297 and 72 nucleotides in length, respectively.

The genomic DNA sequence of *CaMi* shared 98% identity and the deduced amino acid shared 99% identity (Fig. 2) with *Mi-1.2* (accession number: AF039682), the root-knot nematode resistance gene from tomato. However, the intron structures have a striking dissimilarity between these two genes. Intron 1 and intron 2 are both shorter in *CaMi* (1,297 and 72 bp) than in *Mi-1.2* (1,306 and 75 bp), with the sequence identities of 92 and 80%, respectively.

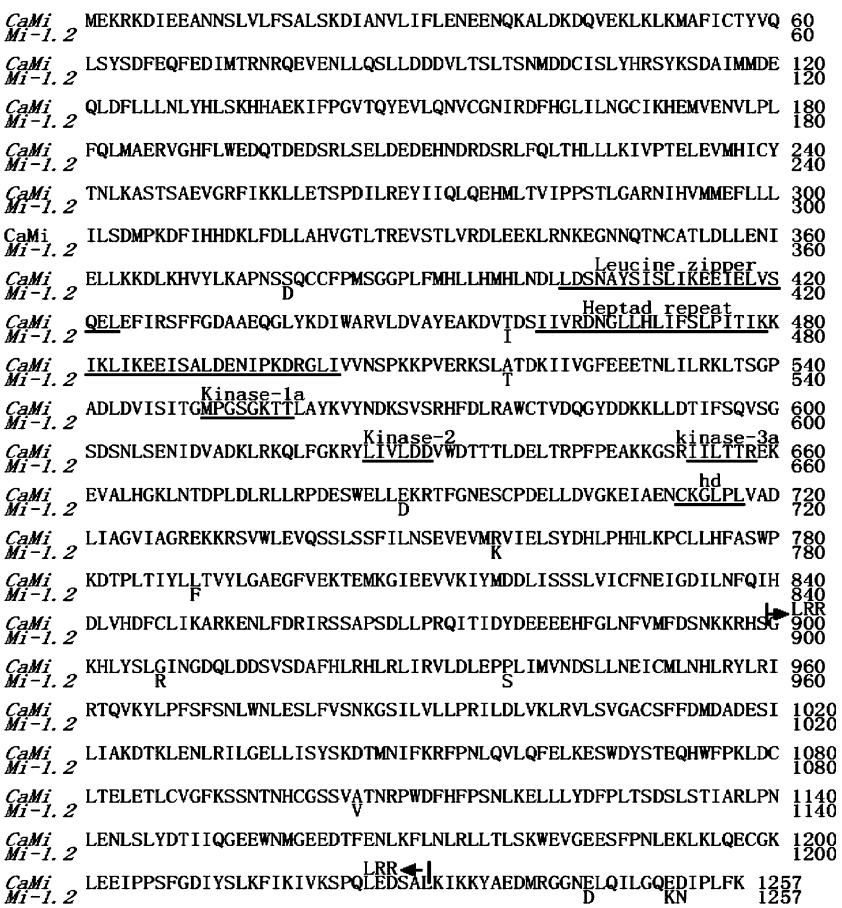


**Fig. 2** Comparison of the predicted amino acid sequences of *CaMi* with *Mi-1.2*, a root-knot nematode resistance gene from tomato (Accession number: AF039682). The deduced amino acid sequence of the *CaMi* gene product is shown and the amino acids that differ from the *Mi-1.2* gene product are indicated. The positions of a potential leucine zipper and a heptad repeat motif are underlined and the boundaries of LRR region are indicated. The kinase-1a, kinase-2, kinase-3a and hydrophobic domain (*hd*) of a predicted nucleotide binding site domain are *underlined*

## Southern blot analysis of *CaMi* in pepper

It has been reported that many isolated *R* genes appear to reside in local multigene families (Jones 2001). In this study, Southern blot analysis was performed to investigate the copy number of *CaMi* in the root-knot nematode resistant pepper line, PR 205. The probe was a 900 bp fragment of the *CaMi* gene. The hybridization pattern indicated that there might be three copies of *CaMi* in the PR 205 genome (Fig. 3). This pattern also suggested that *CaMi* belonged to a small gene family.

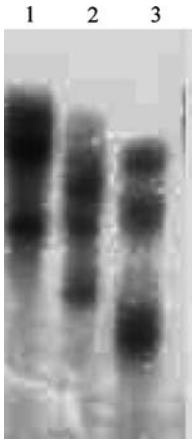


**Fig. 3** Southern blot analysis of *CaMi* from PR 205 of pepper. DNA from root-knot nematode resistant pepper line PR 205 was digested with *Eco*RV (*lane 1*), *Eco*RI (*lane 2*) and *Hin*dIII (*lane 3*), as ordered. The probe was a 900 bp *CaMi* fragment

## Generation and confirmation of transgenic plants

The nematode susceptible tomato lines of ZS-5 and J-8 were transformed with *A. tumefaciens* carrying the sense construct pBCaMi, a binary vector containing *CaMi* genomic DNA covering the full coding region, driven by the cauliflower mosaic virus (CaMV) 35 S promoter. In total, 19 independent putative transformants were obtained and the presence of transgene in 16 independent transgenic lines was confirmed by PCR and Southern blot analysis. The Southern blot result indicated that one to five copies of T-DNA were integrated into the genome of transgenic plants (Fig. 4). In addition, several transgenic plants had the same band patterns (e.g. TJC3 and TJC7; TJC4, TJC5 and TJC9), indicating they were probably derived from the same transgenic bud.

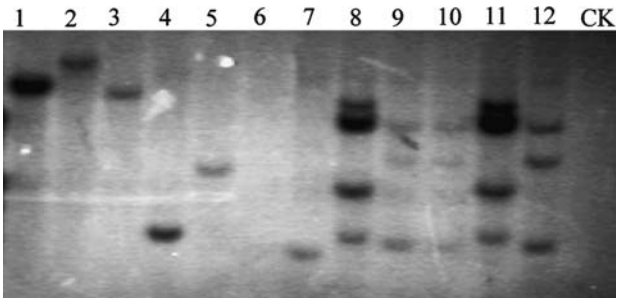


**Fig. 4** Southern blot analysis of genomic DNA from 12 selected $T_0$ tomato *CaMi* transformants. Each lane contains 15 μg genomic DNA extracted from leaves following digestion with *Eco*RV. The probe was a 740 bp *NPT*II fragment. *Lane CK* untransformed tomato ZS-5 DNA, *Lanes 1–12* putative $T_0$ generation tomato transformants TZC1, TZC2, TZC4, TZC6, TZC8, TJC1, TJC2, TJC3, TJC4, TJC5, TJC7, TJC9, respectively

## Expression profiling of *CaMi* in resistant pepper plants and transgenic tomato plants

RT-PCR analysis indicated that *CaMi* was highly expressed in the roots, leaves and flowers, weakly expressed in the stems, and was undetectable in the fruits of the resistant pepper line PR 205 (Fig. 5a).

Analysis of the transgenic tomato plants showed wide variation in the expression of *CaMi* transegene in different transgenic tomato plants (Fig. 5b). The expression level of *CaMi* in the transgenic plants TZC2 and TZC4 was significantly higher than that in other transgenic plants.

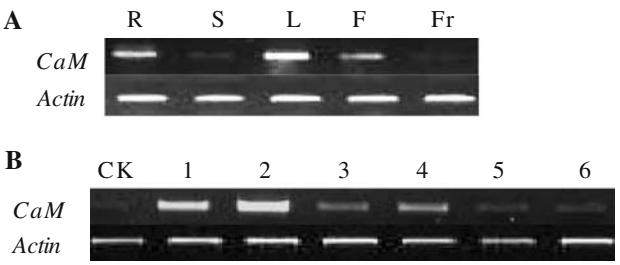


**Fig. 5** RT-PCR analysis of the expression of *CaMi* in resistant pepper line PR 205 and in transgenic tomato plants. **a** Expression pattern of *CaMi* in resistant pepper line PR 205. *R* roots, *S* stems, *L* leaves, *F* flowers, *Fr* fruits. **b** expression of *CaMi* in young leaves of transgenic tomato plants. *Lanes 1–6* TZC2, TZC4, TJC2, TJC3, TJC5, TJC7. *Lane CK* untransformed tomato ZS-5

## Nematode resistance assay of the transgenic tomato plants

Five cuttings of each independent primary transformed plant were tested for resistance to root-knot nematode *M. incognita*. After nematode infection, there were numerous root knots formed in the root system of susceptible tomato line ZS-5 (Fig. 6b), but none was found

in the roots of resistant pepper line PR 205 (Fig. 6c). Among the 12 tomato transgenic plants chosen for testing, all but three were highly resistant to *M. incognita* (Table 2; Fig. 6a). Among the three susceptible transformants, TJC1 had no T-DNA insert while TJC3 and TJC7 were probably derived from the same transgenic plant with four copies of T-DNA insert. This result demonstrated that the *CaMi* gene was sufficient in conferring resistance to root-knot nematodes after it was introduced into nematode-susceptible tomato plants.

The resistance to root-knot nematodes was further tested in plants of one $T_1$ progenies and homozygous plants in $T_2$ lines together with two susceptible tomato lines ZS-5 and J-8, as well as the pepper resistant line PR 205. The results indicated that of the $T_1$ progenies from transgenic tomato plants TZC4, which carried one copy of the introduced T-DNA, 20 were resistant to root knot nematodes and 6 were susceptible. It showed the expected segregation ratio (3:1) in a single dominant gene fashion, and no segregation was found in $T_2$ lines. PCR analysis indicated a correlation between nematode resistance and the presence of T-DNAs of the tested plants (Fig. 7).

**Table 2** Analysis of tomato transgenic plants, which contained *CaMi* gene for nematode resistance

| Transformant[a] | Mean ± SD[b] | Resistance[c] | Copies[d] |
|---|---|---|---|
| TZC1 | 6 ± 2 | R | 1 |
| TZC2 | 3 ± 1 | R | 1 |
| TZC4 | 1 ± 1 | R | 1 |
| TZC6 | 5 ± 2 | R | 1 |
| TZC8 | 7 ± 3 | R | 1 |
| TJC1 | >100 | S | 0 |
| TJC2 | 8 ± 2 | R | 1 |
| TJC3 | 27 ± 5 | S | 4 |
| TJC4 | 19 ± 11 | R | 3 |
| TJC5 | 21 ± 7 | R | 3 |
| TJC7 | 34 ± 9 | S | 4 |
| TJC9 | 15 ± 8 | R | 3 |
| ZS-5 | >100 | S | – |
| RN-1 | 0 | R | – |
| PR 205 | 0 | R | – |

[a] ZS-5 and RN-1: root-knot nematode susceptible and resistant tomato lines, respectively; PR 205: root-knot nematode resistant pepper line

[b] Number of egg masses (mean ± SD). Each data point represents the average of five replicated tests

[c] Plants were classified as resistant (R), if the individual root system had less than 25 egg masses, or susceptible, if the individual root system had 25 or more egg masses

[d] Number of copies of inserted T-DNA as determined by Southern blot analysis

## Ultrastructure changes of roots after nematode infection

The ultrastructure of infected root tissues of tomato transgenic plants and the susceptible line ZS-5 were used for paraffin analysis. In ZS-5, the invading juveniles established a permanent feeding site in the differentiation zone of the roots by inducing nuclear division without cytokinesis in host cells. This process gave rise to large and multinucleated giant cells (Fig. 8a). Hyperplasia and hypertrophy of the surrounding cells led to the formation of the galls or root knots (Fig. 7b). However in the roots of transgenic plants showing resistance to *M. incognita*, the multinucleate giant cells, as well as the galls or root knots, could not be found after nematode infection. In addition, many necrotic cells were found around nematodes (Fig. 8b).

## Discussion

Several R-genes targeted against nematodes have been described and a number of these have been cloned. The first R-gene cloned was $Hs1^{pro-1}$ isolated from *Beta*


**Fig. 6** Nematode resistant transgenic tomato plant, tomato line ZS-5 and pepper line PR 205. **a** The root of tomato transgenic plant over-expressing *CaMi* that showed resistance to root-knot nematodes. **b** The root of tomato ZS-5 which is susceptible to root-knot nematodes. *Arrows* indicate root-knots in the infected root. **c** The root of pepper PR 205 which is resistant to root-knot nematodes

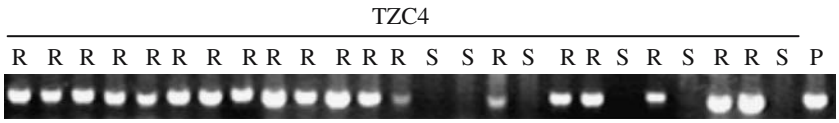


**Fig. 7** PCR analysis of the progenies of tomato transgenic lines TZC4. Progenies of tomato transgenic line TZC4, in which the expression of *CaMi* was elevated, were assayed for nematode resistance. The presence of the introduced *CaMi* gene in these progenies was detected by PCR using the primers SP1 and SP2. The expected band was present in all resistance plants (*R*) but not in any susceptible plants (*S*). *Lane P* contains DNA from the primary transformant TZC4

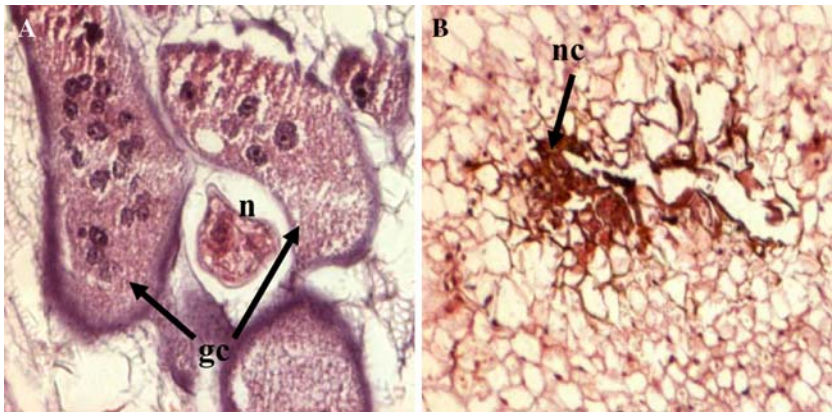


**Fig. 8** Root microscopy analysis of the tomato after infected by *M. incognitia*. **a** Cross-section analysis of the roots of tomato ZS-5, which is susceptible to *M. incognitia*. The nematodes had induced several giant-cells. **b** Cross-section of the roots of tomato transgenic plants over-expressing *CaMi*. After nematodes penetrated into the roots, it elicited hypersensitive response (*HR*) and produced cell death around the nematode. *n* Nematode, *gc* giant cell, *nc* necrotic cell

*procumbens*, a wild species of sugar beet that confers resistance to the cyst nematode *Heterodera schachtii* (Cai et al. 1997). This gene is coding an acidic protein component of 282 amino acids and it differentially expressed in root. Transfer of the *Hs1pro-1* gene to a susceptible beet line conferred nematode resistance on hairy roots.

The second resistance nematode gene was *Cre3*, which conferred resistance to cereal cyst nematode in wheat (*Triticum aestivum* L.) (Lagudah et al.1997). Cre3 belongs to the style of NBS–LRR, it specially expressed in wheat root. van der Vossen et al(2000) separated a resistance potato cyst nematode *Globodera pallida* gene *Gpa2* from potato, it belongs to LZ–NBS–LRR family. Another nematode resistance gene that has been cloned is the *Hero* gene of tomato (Ernst et al 2002). *Hero* confers wide-spectrum resistance to potato cyst nematodes, displaying 95% resistance to *Globodera rostochiensis* and over 80% resistance to *G. pallida*. Except *Mi*, other resistance to root knot nematode genes has not been cloning and they had only located on the chromosome (Ammiraju et al. 2003). Especially, there is no report about the cloning of root knot nematode gene in pepper.

Gene isolation, based on the homologue sequence in closely related species has been proved to be a quick and convenient approach. It is accomplished by PCR strategies with specific primers, which is normally designed through the comparison of known gene sequences that are homologues to the desired gene for cloning. In this study, we designed degenerate primers according to the sequence of the four highly conserved domains (Kinase-1a, Kinase-2, Kinase-3a and hydrophobic domain) of the *R* genes from different plant species. We were able to successfully clone a root-knot nematode resistance gene, *CaMi*. The full-length cDNA sequence and genomic DNA sequence of *CaMi* were also determined in this study.

Among the members of the *Solanaceae* family, tomato and pepper have very close phylogenetic relationships and structural genomic homologies (Castaagnone-Sereno et al. 1992). Several single dominant genes conferring resistance to root-knot nematodes have been discovered in both tomato and pepper. The *CaMi* from hot pepper and *Mi* gene from tomato can trigger tissue necrosis or hypersensitive response (HR) at the site of the root-knot nematode invasion (Fig. 8b) and subsequently prevent juvenile invasion,

causing the juveniles to die or leave the host roots, thus conferring resistance to root-knot nematodes. In addition, these two genes have the same gene structure. Both of them have two introns and uniform intron locations. The deduced open reading frames of both genes encode for 1,257 amino acids and the amino acid sequences shared 99% identity. The above findings suggest that it is highly possible that these two genes are derived from the same ancestor. Besides conferring resistance to root-knot nematodes, the *Mi* gene also confers resistance to some isolates of the potato aphid (*Macrosiphum euphorbiae*) and the sweet potato whitefly (*Bemisia tabaci*) (Rossi et al., 1998; Nombela et al. 2003). Whether the *CaMi* gene has the ability in conferring the same resistance requires further study.

A number of root-knot nematode resistance genes have been discovered in *Capsicum annuum* L., while the resistance spectrum and genetic properties of these genes are quite different. Hare (1956) identified a dominant resistance gene *N* in *C. frutescens* L. that has efficient resistance to the three main root knot nematodes, but its ability to confer resistance is rendered ineffective at temperature over 28°C. On the other hand, studies of homozygous progenies obtained by androgenesis showed that at least five genes (*Me1*, *Me2, Me3, Me4*, and *Me5*) are involved in root-knot nematode resistance while they differ in their range of activities against the *Meloidogyne* species and population, as well as by their phenotypic expression in the roots (Hendy et al. 1985). Two of them (*Me1* and *Me3*), which are not alleles, are active against the same species as the *Mi* tomato gene (Hendy et al. 1985). In this study, the *CaMi* gene is also active against the same species as the *Mi* gene. Whether *CaMi* is one of the previously discovered root-knot nematode resistance genes needs further research.

In susceptible tomato lines the root-knot nematode resistance gene, *Mi*, is associated with an inverted chromosomal segment (Seah et al. 2004). However, in pepper, we were not able to determine this kind of inversion. When using the susceptible pepper DNA as template, we failed to obtain any bands with the primers SP5 and SP6 by PCR amplification (data not shown). A possible explanation for this observation is that either the fragment of *CaMi* gene is not present in the genome of susceptible pepper lines or this gene developed mutations, thus losing its function of resistance to root-knot nematodes.

During the past decade, researchers worldwide have made significant progress towards pepper regeneration and transformation (Manoharan et al. 1998). However, tissue culture techniques in pepper still lag behind other *Solanaceous* species mainly due to its recalcitrance to regeneration (Ochoa-Alejo and Ramirez-Malagon 2001). The production of phenotypically abnormal plants with distorted or rosette of distorted leaves without normal shoots have been a major constraint in the plant regeneration reported for pepper (Hyde and Phillips 1996). In tomato, the root-knot nematode gene *Mi* was reisolated from tomato and introduced into the susceptible tomato lines ZS-5 and J-8 by Chen et al (2006). It was successful to obtain the nematode resistant transgenic plants. So in this study, due to the lack of a reliable system for pepper regeneration and transformation, *CaMi* was over-expressed in tomato, which has routine transformation technology, to gain insight into its possible function in root-knot nematode resistance. When the susceptible tomato cultivars over-expressed this gene, they have an efficient resistance to root-knot nematodes. In the transgenic plants, there are no or a few egg-masses on the roots after nematode infected (Table 2; Fig. 6). These results suggest that the transgenic method is a feasible approach to improve the root-knot nematode resistance. In addition, the approach described in this study offers a new way for breeders to generate resistant varieties for other crops that can be seriously damaged by root-knot nematodes and for which no genetic resources of resistance have been identified.

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