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Full Length Research Paper

Characterization of the *psoRPM1* gene for resistance to root-knot nematodes in wild myrobalan plum (*Prunus sogdiana*)

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Several root-knot nematode (*Meloidogyne* spp.) resistance genes have been discovered in different stone fruit crops. However, none of them has yet been cloned and they were only located on the chromosomes. In this study, a candidate root-knot nematode resistance gene (designated as *psoRPM1*) was isolated from the individual plant of Xinjiang wild myrobalan plum (*Prunus sogdiana*) by degenerate PCR amplification combined with the RACE technique. The gene had a typical NBS-LRR structure and high homology with *Mi-1.2* (root-knot nematode resistance genes in tomato). The expression of *psoRPM1* gene increased in the roots of resistant wild myrobalan plum material 12, 24 and 48 h after inoculation with root-knot nematodes and the expression of *psoRPM1* gene was maximum 12 h after inoculation. But in susceptible plant, the *psoRPM1* gene expression remained low both before and after inoculation. This result suggested that the *psoRPM1* gene was constitutively expressed gene in the wild myrobalan plum. *In-situ* hybridization results showed that the *psoRPM1* gene mainly expressed in both phloem and cortex parenchyma of root 12 h after inoculation in resistant plant. Furthermore, the *psoRPM1* gene only expressed in phloem 48 h after inoculation in resistant plant. The result suggested that the *psoRPM1* gene played a role in keeping nematodes off the cortex when nematodes began to infect the plant's roots. After root-knot nematodes entering into cortex parenchyma, the *psoRPM1* gene mainly played defense function in phloem of pericycle. Using the gene gun bombarding into onion epidermal cells, the result was that *psoRPM1* protein was located in cytomembrane and might be interacted with other proteins in cytomembrane to locate

Key words: Xingjiang wild myrobalan plum (*Prunus sogdiana*), root-knot nematodes (*Meloidogyne incognita*), gene, *in-situ*, gene location.

INTRODUCTION

The root-knot nematode (*Meloidogyne* spp.) is a sedentary parasite of plant roots in many economically important cropping systems where they cause severe yield loss (Williamson and Hussey, 1996). Among them, the root-knot nematode (*Meloidogyne incognita*) is a devastating pathogen to many horticulture plant species, especially in fruits and vegetables. Currently, the primary methods to control root-knot nematodes are crop rotation (Dong et al., 2007), soil fumigation (Bridge, 1996) and chemicals (Onifade et al., 2008). However, these technologies for nematode control have left much to be desired, such as fumigation. Fumigants are poorly environmental and many have been restricted in use. And perennial fruit trees rotation can hardly be achieved. Cultivars resistant to root-knot nematodes can potentially reduce environmental pollution and toxic systemic nematicides are unnecessary as they won't be an efficient and durable control method (Djian-Caporalino et al., 1999). So, cloning and functional verification of resistant gene

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Figure 1. The susceptible (A) and resistant (B) plants of the wild myrobalan plum.

(R-gene) is an effective way to achieve transgenic breeding (Williamson and Kumar, 2006). Cloning the R-genes and genetic transformation is one of the most important objectives in breeding program and is particularly relevant in fruit tree crops where generation time and population size hamper rapid breeding response to pathogens and pests.During the past 15 years, over 70 *R* genes have been cloned from several different plant species and some of them have been well characterized (Milligan et al., 1998; Ferrier-Cana et al., 2003), but few of them are genes resistant to root-knot nematodes (RKN). In horticulture plants, one of the best characterized crops for nematode resistance is tomato. The tomato *Mi*-1.2 gene (Vos et al., 1998) encodes a leucine-rich repeat protein and confers resistance to three *Meloidogyne* species as well as aphids and white flies. *Mi*-1.2 can be transgenically expressed and provide *Meloidogyne* resistance in susceptible tomato as well as relationship plant species (Goggin et al., 2006). But *Mi* - 1.2 gene does not confer resistance against the same nematode when introduced into tobacco or *Arabidopsis* (Williamson and Kumar, 2006). In *Prunus* species, different ranges of resistance to root-knot nematodes (RKN) have been observed and corresponding genes have been used for rootstock breeding (Esmenjaud et al., 1997). Now only four RKN resistance genes in *Prunus* rootstock material, the *Ma*, *Rjap*, *RMia* and *MJ* genes in Myrobalan, Japanese plums, peach and almond respectively, have been identified and validated by molecular markers (Lecouls et al., 2004; Claverie et al., 2004; Ghelder et al., 2010). But full-length DNA or cDNA sequences of these genes and transgenic breeding have not been carried out. And the mechanism of RKN resistance in *Prunus* spp. has been poorly investigated. Therefore, it is necessary to explore and screen the RKN R-gene from different fruit tree species to expand the genetic basis and prevent the root-knot nematode diseases to a greater degree.

The Xinjiang wild myrobalan plum (*Prunus sogdiana*) distribute in the Tianshan Mountains area from the Kazakhstan-Chinese border. The Ily River originates in the western Tianshan Mountains in China and flows northeastward, then westward to Kazakhstan and finally, drains into the Balkhash Lake. A large area of wild myrobalan plum (*P. sogdiana*) covers the slopes along the river and its branches (Romero et al., 2003). Wild myrobalan plum shows good characterization of resistance to root-knot nematode (*M. incognita*) and has generated useful genetic materials to engineer novel resistant cultivars (xiao et al., 2010). However, the research of molecular biology character for Xinjiang wild myro-balan plum remains poorly documented. In this study, we used the individual plant that was resistance to root-knot nematodes as experimental material and a candidate root-knot nematode resistance gene (*psoRPM1*) was cloned by degenerate PCR amplification combined with the RACE technique. The researches of *psoRPM1*gene's structure, expression and localization, would provide a starting point for understanding the mechanism of RNK resistance in Wild myrobalan plum. And this gene could be introduced into many other stone fruit crops that could be seriously damaged by root knot nematodes.

MATERIALS AND METHODS

Plant materials

Wild myrobalan plum (*P. sogdiana*) was introduced from Xinjiang, China and then planted in Shangzhuang experimental station of China Agricultural University. Second-stage juveniles (J2) of nematode that had been hatched within a 24 h period were collected from a hydroponic culture system as inoculums (Lambert et al., 1992). Wild myrobalan plum was inoculated with nematodes according to the procedure described by Yaghoobi et al. (1995). Plants were infected with approximately 3,000 J2 of nematodes in the third month after cuttage. Roots were harvested 6 to 8 weeks later to observe the number and size of root-knot. The most resistant and susceptible plants (Figure 1) were chosen among all materials and the most resistant plant was used to clone the R-gene.

Cloning of *psoRPM1* gene and structure analysis

RNA was extracted from young roots of resistant material using the method described by Salzman et al. (1999). According to consensus analysis of conserved motif of various resistant (R) genes, we obtained degenerate primers DP1 and DP3 (Table 1, All the primes of this article are described in Table 1). Wanted intermediate fragment was obtained from the amplification products

Table 1. Primer sequences used in this study.

Primer	Sequence
DP1	5'-GGNDYMGGBAAAACTACTCT-3'
DP3	5'-TRCNATNGCNAGNGGNAGNCC-3'
GSP1	5'-CCAGATAGACAGCTTGGAAGTCGAATC-3'
GSP2	5'-GCCATGTTCACTGTATGCAACCCTTGG-3'
GSP3	5'-CAAGCTCCGAAACAAGTCTTCAATCACA-3'
GSP4	5'-TTTGGGAGACACTGATCCAAGCATAAGA-3'
SP1	5'-ATGGACTCAGCTCCAACAGCTCTCT-3'
SP2	5'-TCATGTGGGTGAACTATGATGAGCT-3'
18SrRNA-F	5'-AGCAGAACGACCCGAGAA-3'
18SrRNA-R	5'-CCGAGGACTTGGCATTTA-3'
psoRPM1-F	5'-TCTCCCAAACTTATGTGATT-3'
psoRPM1-R	5'-CGACTTCCAAGCTGTCTAT-3'
pZES-F	5'-GTCGACATGGACTCAGCTCCAACAG-3'
pZES-R	5'-GGATCCAATGTTTTCCCAGATCCTCC-3'
YW-F	5'TGGATCCTCTCCCAAACTTATGTGATT3'
YW-R	5'TGAAGCTTCGACTTCCAAGCTGTCTAT3'

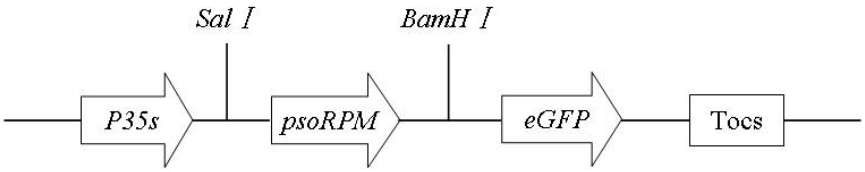

Figure 2. Schematic map of *psoRPM1*-GFP construction.

of the two primers. Using the intermediate fragment, two primers,GSP3 and GSP4 were designed. Then 5'RACE products were obtained by the two-step method. In the same way, 3'RACE products were eventually got after designing the two primers, GSP1 and GSP2. Matching the 3'RACE products, 5'RACE products and intermediate fragments, we obtained the full-length cDNA of candidat root-knot nematode R-gene *psoRPM1*. DNA was extracted from young leaves of resistant plant material using the method described by Fulton et al. (1995). According to the full-length cDNA, we designed two primes SP1 and SP2, and then obtained the full-length DNA of *psoRPM1*. We analyzed open reading frames and found out all kinds of nematode R-genes in Genebank database. The homology of these genes was compared using MEGA4.0 software and sequences were aligned with DNAMAN software.

RT-PCR analysis

The resistant and susceptible myrobalan plum was chosen to inoculate with root-knot nematodes. Total RNA was isolated from roots 12, 24 and 48 h after nematode inoculation as well as from non-inoculated plants. RT-PCR was performed with gene-specific primers: psoRPM1-F and psoRPM1-R.

Probe preparation and *in-situ* hybridization

The *psoRPM1* probe (150 bp long) was obtained with primes YW-F and YW-R, which were designed according to the cDNA sequence. PCR was performed using total RNA and then PCR products were sequenced. The correct fragment was digested by restriction enzymes *BamHI* and *HindIII* (MBI, Fermatas) and then the product was inserted into pSPT-18 vector (Roche Applied Science, Penzberg, Germany) to get plasmid. This plasmid was digested with *BamHI*. According to the DIG labeled RNA probe light kit (SP6/T7), antisense probe was synthesized, while sense probe was synthesized as control.

The roots of resistant material, which were inoculated after the12, 24 and 48 h, and non-inoculated, were used for *in-situ* hybridization. Materials were fixed in 4% paraformaldehyde (PFA) for 15 h at 4℃ and then dehydrated in a graded ethanol series (30, 50, 70, 85 and 95%). The materials were embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO, USA). Ten-micrometer slices were cut with a microtome and mounted on glass slides. The hybridization signals were observed and recorded by light microscopy (BX61 Olympus, Tokyo, Japan) (Bowman et al., 1991).

Plasmid construction and subcellular localization

A pair of primers was used to amplify *psoRPM1*. The primers sequence were 5'-GTCGACATGGACTCAGCTCCAACAG-3' (*SalI* cutting site added) and 5'-GGATCCAATGTTTTCCCAGATCCTCC-3' (*BamHI* cutting site added). The PCR product was fused with the green fluorescent protein (GFP) in the C-terminus and cloned into the pEZS-NL vector under the control of the 35S promoter (Figure 2). Transient expression of the CaMV 35S:: *psoRPM1*-eGFP fusion construct and the CaMV 35S::eGFP control was performed by

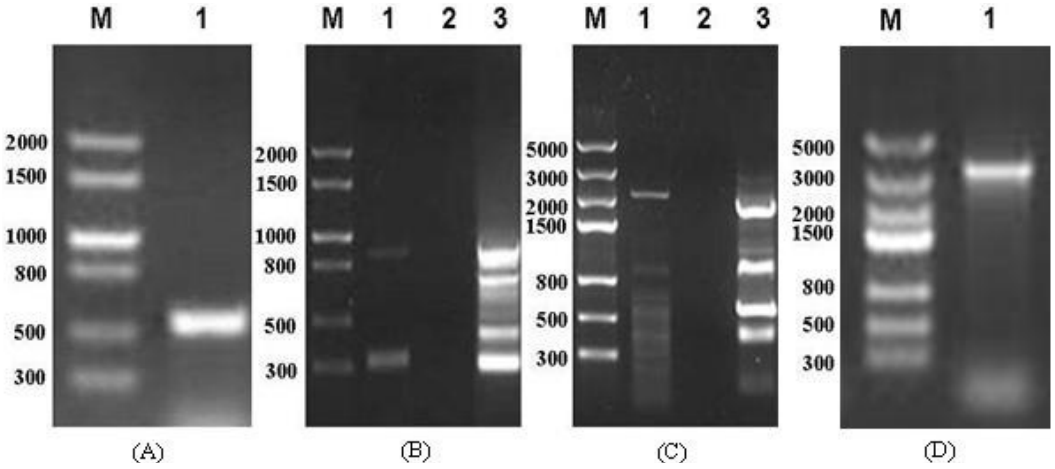

Figure 3. Cloning the root-knot nematode candidate R-gene *psoRPM1* in wild Myrobalan plum. (A) Amplification of intermediate fragments; (B) amplification of 5' region; (C) amplification of 3' region; (D) the full-length DNA of *psoRPM1*.

introducing the resultant plasmids into onion (*Allium cepa L.*) epidermal cells by means of the particle bombardment method according to the manufacturer's protocol (Scott et al., 1999). The transformed cells were cultured on MS medium at 25°C for 24 h and observed under a Bio-Rad MRC-1024 confocal laser scanning microscope (Bio-Rad, CA, USA) for detecting the fluorescence (Nigam et al., 2008).

RESULTS

Isolation of *psoRPM1* and sequence analysis

The individual plant resistance to root-knot nematodes was used to isolate the *psoRPM1*gene. A 537 bp intermediate fragment was obtained by the degenerate primers DP1 and DP3 (Figure 3a). The full-length cDNA sequence of *psoRPM1*was obtained using RACE amplification. We obtained the 5′region of 871 bp (Figure 3b) and 3′region of 2119 bp (Figure 3c). The full-length cDNA of *psoRPM1*was 3226 bp, which included a potential open reading frame of 2754 bp, a 5′-untranslated region (5′-UTR) of 193 bp and a 3′-untranslated region (3′-UTR) of 279 bp, including the poly (A) tail. According to the full-length cDNA sequence, we designed the specific primers SP1 and SP2. The genomic DNA was isolated from the individual plant of resistant to root-knot nematodes using primers SP1 and SP2 and the full-length DNA of putative root-knot nematode R-gene was 3226 bp too (Figure 3d). Named it *psoRPM1* gene, with an accession number of HM593974 in GenBank database.

The potential open reading frame of *psoRPM1*encoded for 917 amino acid residues and contained a nucleotide binding site (NBS) domain and a leucine-rich repeats (LRRs) domain. The predicted NBS domain was comprised of the P-loop, kinase-2, kinase-3 motif and hydrophobic domain (hd). At the C terminus, it contained a leucine- rich repeats (LRRs) domain. The amino acids sequence of *Mi-1.2* also contained the NBS and LRRs conserved motifs. The NBS motifs of *Mi-1.2* and *psoRPM1* were entirely consistent, but in the LRRs motifs there were many differences between *Mi-1.2* and *psoRPM1* which contained a lot of leucine repeat sequences (Figure 4).

Phylogenetic analysis of the *psoRPM1* gene

To estimate the phylogenetic relationship between *psoRPM1* and known nematode R-genes, one neighbour-joining phylogenetic tree was constructed. Based on the degree of similarity among of amino acid sequences, 16 RKN genes were classified in three subfamilies (designated as I, II and III) (Figure 5). The highest similarity was detected between *psoRPM1* and four genes, two hot peppers (ABE68835.1 and DQ465824.1) and two tomatoes (AAC67238.l and AF039682.1), one of which was *Mi*-1.2; the first discovered root-knot nematode R-gene from tomato. These five genes were grouped in I subfamily. It is worth pointing out that *psoRPM1* and *Mi-1.2* shared high similarity. Other nine R-genes were grouped in II and III subfamilies, showing that these R-genes were not very similar with *psoRPM1*.

The *psoRPM1* gene expression in root

The RT-PCR analysis of the *psoRPM1* gene of resistant and susceptible plants is shown in Figure 6. Before inoculation with root-knot nematodes, the expression of *psoRPM1* was very low in resistant and susceptible plants. But 12, 24 and 48 h after inoculation, the *psoRPM1* gene expression levels were significantly increased in resistant plant. And the expression reached

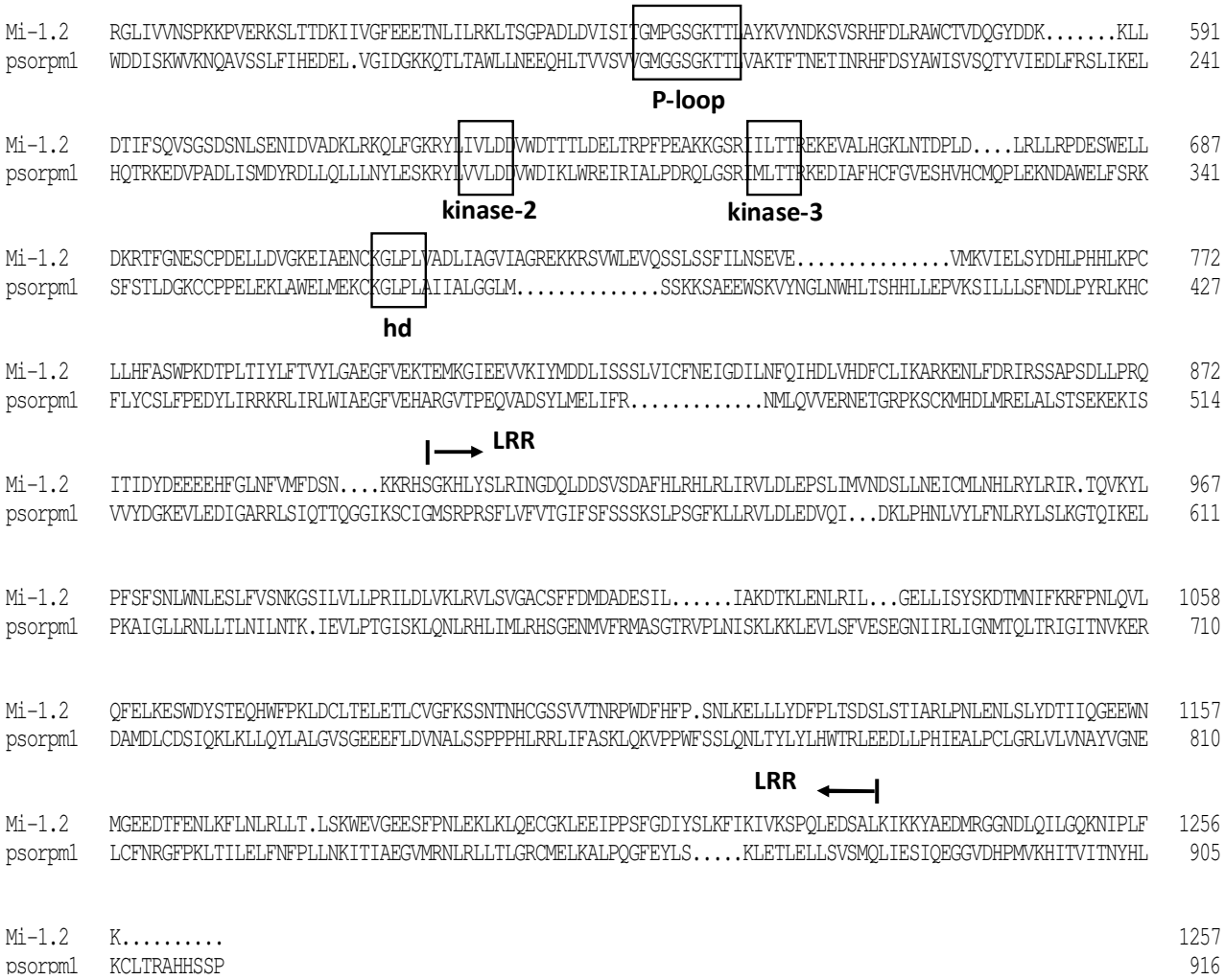

Figure 4. Comparison of the predicted amino acid sequences of *psoRPM1* with *Mi*-1.2, a root-knot nematode resistance gene from tomato (accession number: AAC67238.1). The positions of LRRs region are indicated. The P-loop, kinase-2, kinase-3 and hydrophobic domain (hd) of a predicted nucleotide binding site domain are showed.

highest level at 12 h after inoculation. However, there were no notable changes in *psoRPM1* gene expression in susceptible plant during any period of time after inoculation and the *psoRPM1* gene expression was still very low (Figure 6) . *In-situ* hybridization results of *psoRPM1* gene in resistant individual plant root are shown in Figure 7. Before inoculation with root-knot nematodes, the *psoRPM1* gene was weakly expressed in pericycle of root (Figure 7a). 12 h after inoculation, the *psoRPM1* gene was expressed strongly in pericycle and cortex of root (Figure 7b) and the expression signal mainly accumulated in the primary phloem of root (Figure 7c). 24 h after inoculation, the *psoRPM1* gene become weakly in the cortex of root (Figure 7d), while the *psoRPM1* hybridization signal were still strong to be detected in the primary phloem of root (Figure 7e). 48 h after inoculation, the signal was not detected in the cortex of root (Figure 7f), but in the primary phloem the *psoRPM1* gene were still strongly expressed (Figure 7g).

Subcellular localization of *psoRPM1* protein in onion epidermal cells

To determine the cellular localization of the *psoRPM1* protein, the *psoRPM1* was fused into the pEZS-NL vector, downstream of a constitutive CaMV35S promoter and upstream of a eGFP gene to create a CaMV 35S::*psoRPM1*-eGFP fusion construct. It was subsequently introduced into the onion epidermal cells by particle bombardment. After incubation for 24 h, the control, transformed with CaMV 35S::eGFP construct alone, exhibited intact cell under visible light (Figure 8a). In the dark field (UV light), the GFP signal distributed in the plasma membranes and all cytoplasm of onion epidermal cells (Figure 8b). In the overlay images of visible light and UV light, the GFP signal was also detected in the entire cells (Figure 8c). By contrast, the onion epidermal cell was intact in the visible light field too (Figure 8d) and eGFP fluorescence was detected and

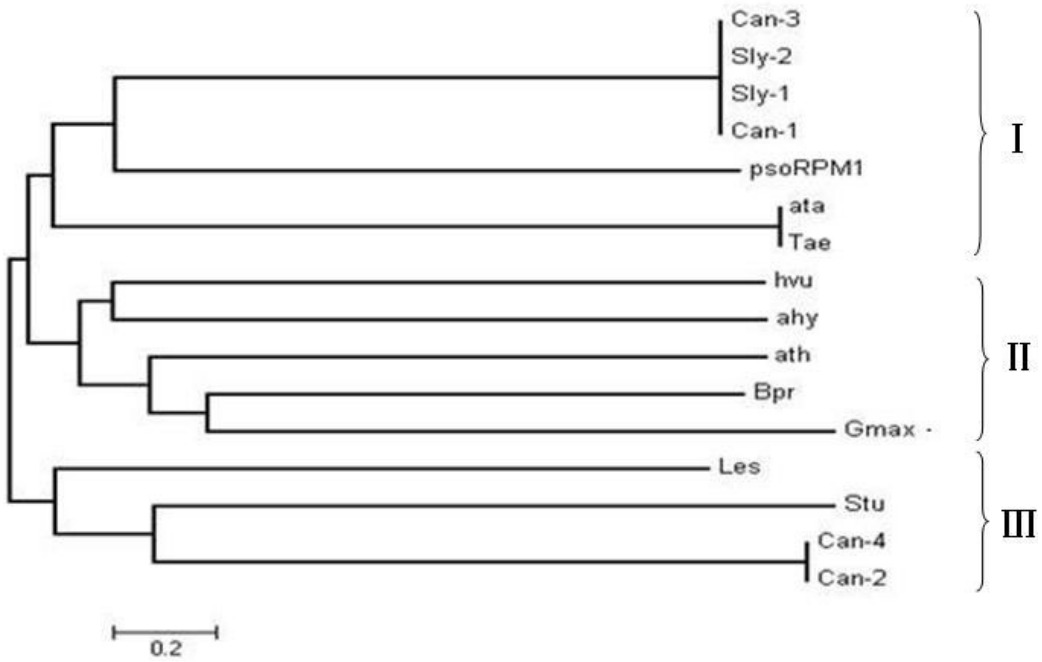

Figure 5. Phylogenetic tree of the *psoRPM1* gene and R-gene in different plant species. Phylogenetic tree based on the amino acid alignment of the consensus sequences along with cloned R genes:Can-3 (*Capsicum annuum* DQ465824.1), Sly-2 (*Lycopersicon esculentum* AF039682.1), Sly-1 (*Solanum lycopersicum* AAC67238.1), Can-1 (*Capsicum annuum* ABE68835.1), *psoRPM1* (*Prunus sogdiana*), ata (*Aegilops tauschii* AF052641), Tae (*Triticum aestivum* EU327996.1), hvu (*Hordeum vulgare* AAG30254.1), ahy (*Arachis hypogaea* AAW55559.1), ath (*Arabidopsis thaliana* AAK59456.1), Bpr (*Beta procumbens* DQ148271.1), Gmax (*Glycine max* EU836688.1), Les *(Solanum lycopersicum* CAD29728.1), Stu (*Solanum tuberosum* AY196151.1), Can-2 (*Capsicum annuum* ACI43068.1), Can-4 (*Capsicum annuum* FJ231739.1).

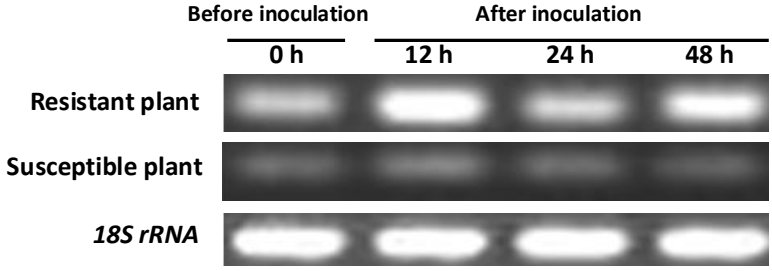

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Figure 6. Expression patterns of the *psoRPM1* gene detected by RT-PCR before and after inoculation with root-knot nematodes

revealed that the fluorescence signal of CaMV 35S:: *psoRPM1*-eGFP fusion protein was predominantly localized on the plasma membranes of that in onion epidermal cells (Figure 8e). When Figure 8d and e were overlapped, the eGFP singal was also localized on the plasma membranes of that in onion epidermal cells (Figure 8f).

DISCUSSION

In stone fruit crops, the most rootstock material is susceptible to root-knot nematodes (RKN) and resistance sources have been sought with the objective of controlling these pests (Kochba and Spiegel-Roy, 1972; Kester and Grassely, 1987; Nyczepir, 1991). The myrobalan plum (*Prunus cerasifera*) expressing resistance to the root-knot nematodes was selected (Salesses et al., 1994). Among them the myrobalan plum accession P.2175 is highly resistant to all tested RKN species (*M. incognita*, *Meloidogyne arenaria*, *Meloidogyne javanica*, *Meloidogyne floridensis*, and *Meloidogyne mayaguensis*) (Rubio-Cabetas et al., 1999), a trait conferred by the major dominant gene *Ma*. In our past research, the

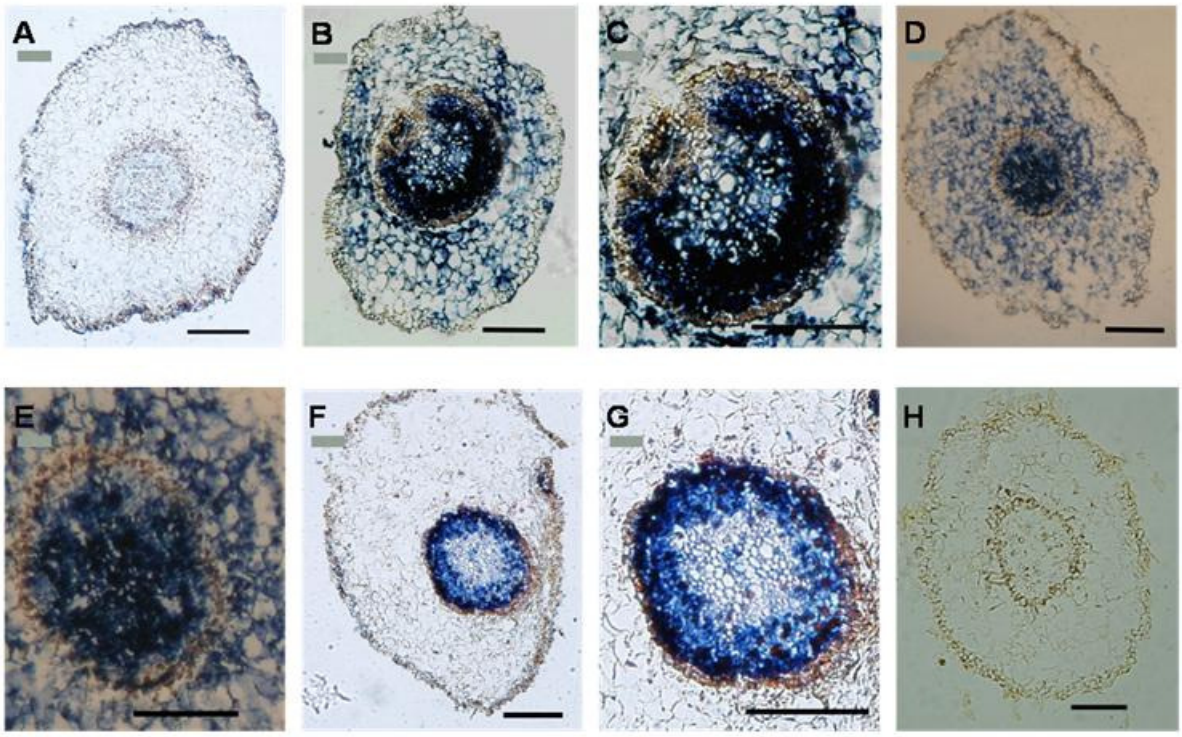

Figure 7. *In-situ* expression pattern of *psoRPM1* gene. A, Before inoculation; B, C, 12 h after inoculation; D, E 24 h after inoculation; F, G 48 h after inoculation; H is the sense *psoRPM1* probe control. The scale bar indicates 200 µm.

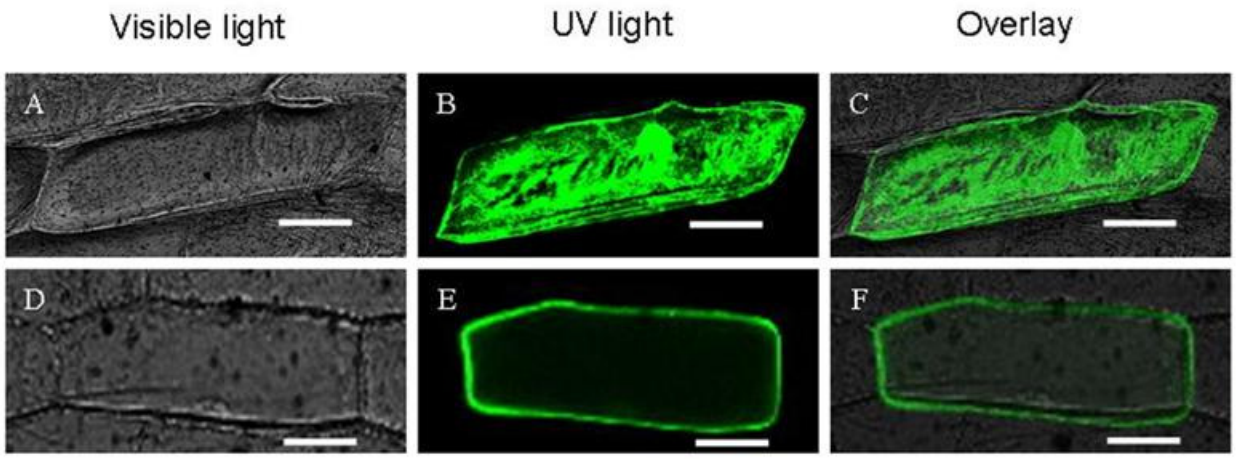

Figure 8. Subcellular localization of CaMV 35S::GFP and CaMV35S::*psoRPM1*-eGFP fusion constructs in onion epidermal cells by transient expression. The photographs were taken in the bright light filed for the morphology of the cell (A and D), in dark field for green fluorescence (B and E) and overlay images (C and F), respectively, for p35S::eGFP control plasmid (A-C) and p35S::*psoRPM1*-GFP plasmid (D-F). The scale bar indicates 100 µm.

Xinjiang wild myrobalan plum (*P. sogdiana*) was one of the RKN resistance sources and resistant to predominant RKN species especially to *M. incognita*, which was most frequent RKN species in China. In our research, highly resistant individual plant was chosen as experimental object to study the resistance mechanism of root-knot nematode.

The structure of proteins which many cloned R-genes

encode contains the nucleotide binding site-leucine rich repeat domain (NBS-LRR), such as *Mi*(tomato), *Gpa2/Rx1* (potato), *Hero*(tomato), *Cre*(wheat) genes, etc. Proteins, these genes encode have a variable N-terminal domain of approximately 200 amino acids (aa), a predicted NBS domain of approximately 300 aa and a more variable tandem array of approximately 10 to 40 short LRR motifs (Ellis et al., 2000). LRR motifs have been found to participate in protein-protein interactions in a wide range of organisms (Kobe and Kajava, 2001). The general NBS-LRR structure is well adapted to recognize a wide range of signals and confers resistance to a number of bacterial, fungal and viral pathogens. NBS - LRR resistance genes have been cloned from a variety of plant species. In this study, the *psoRPM1* belonged to the style of NBS-LRR and there was high similarity between *psoRPM1* and *Mi*-1.2. Therefore, it is presumed that *psoRPM1* may be related to root-knot resistance.

Most RKN R-genes are constitutively expressed in the plant (Thurau, 2003), one of which is Mi-1 (Goggin et al., 2004; Martinez and Kaloshian, 2001). Only 12 h after inoculation of tomato roots with root-knot nematodes, general (nonspecific) plant defense genes were up-regulated (Williamson and Hussey, 1996). The cellular HR (hypersensitive reaction) is associated with the presence of *Mi* occurs near the head of the J2 at approximately 12 h after inoculation, roughly the time when the nematode would be expected to inject stylet secretion to initiate giant cell development (Hewezi et al., 2008). In this research, the RT-PCR result demonstrated that expression of *psoRPM1* was obviously increased in resistant materials after root-knot nematodes treatment, especially 12 h after inoculation. By contrast, there was no obvious variation in the susceptible material after nematodes treatment and the expression of *psoRPM1* was very low. These results suggest that the *psoRPM1* was constitutively expressed and responded very quickly to the infection. When the plant response is too weak or too late, the infection will be successful Gheysen G and fenoll C, 2002). Perhaps up-regulation of some nematode R-genes is required for maintaining or enhancing induction of the signal trans-duction pathways leading to resistance.

The plants have an innate immune response and are dependent on specific plant R-gene that detects the invading nematode (Starr et al., 2002). RKN are generally thought to invade into roots 24 to 48 h after inoculation (Gheysen and Fenoll, 2002). The infective second-stage juveniles (J2) 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). In our study, the expression of *psoRPM1* was considerable in both phloem and cortex parenchyma of root 12 h after inoculation, but only in phloem of pericycle after 48 h inoculation. The result suggests that *psoRPM1* played a role in keeping nematodes off the cortex when root-knot nematodes began to infect the plant's roots. After nematodes entering into cortex parenchyma, *psoRPM1* mainly played defense function to make the nematodes out of access to phloem of pericycle. It was presumed that expression of *psoRPM1* could effectively prevent root-knot nematode from absorbing nutrients to influence its growth and development.

The general NBS-LRR structure can recognize a wide range of signals. A NBS-LRR protein is localized in the plasma membrane and it is termed cellulose binding protein (CBP). The first CBP was MI CBP-1 from the root-knot nematode *M. incognita* (Ding et al., 1998). MI CBP-1 was found to bind to cellulose and plant cell walls, but lacked cellulase activity. It indicated that the cellulose-binding protein would have a role in RNK pathogenesis (Hewezi et al., 2008). In our study, *psoRPM1* protein did not contain a predicted transmembrane segment and signal peptide and was localizated in cytomembrane of onion epidermal cells. These results suggest that the *psoRPM1* protein would be CBP protein and interact with other protein thereby inhibiting and potentially targeting to RNK parasitism.

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