RESEARCH ARTICLE

Novel Pectate Lyase Genes of *Heterodera glycines* Play Key Roles in the Early Stage of Parasitism

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Abstract

Pectate lyases are known to play a key role in pectin degradation by catalyzing the random cleavage of internal polymer linkages (endo-pectinases). In this paper, four novel cDNAs, designated *Hg-pel-3*, *Hg-pel-4*, *Hg-pel-6* and *Hg-pel-7*, that encode pectate lyases were cloned and characterized from the soybean cyst nematode, *Heterodera glycines*. The predicted protein sequences of *HG-PEL-3*, *HG-PEL-4* and *HG-PEL-6* differed significantly in both their amino acid sequences and their genomic structures from other pectate lyases of *H. glycines* (*HG-PEL-1*, *HG-PEL-2* and *HG-PEL-7*). A phylogenetic study revealed that the pectate lyase proteins of *H. glycines* are clustered into distinct clades and have distinct numbers and positioning of introns, which suggests that the pectate lyase genes of *H. glycines* may have evolved from at least two ancestral genes. A Southern blot analysis revealed that multiple *Hg-pel-6*-like genes were present in the *H. glycines* genome. *In situ* hybridization showed that four novel pectate lyases (*Hg-pel-3*, *Hg-pel-4*, *Hg-pel-6* and *Hg-pel-7*) were actively transcribed in the subventral esophageal gland cells. A semi-quantitative RT-PCR assay supported the finding that the expression of these genes was strong in the egg, pre-parasitic second-stage juvenile (J2) and early parasitic J2 stages and that it declined in further developmental stages of the nematode. This expression pattern suggests that these proteins play a role in the migratory phase of the nematode life cycle. Knocking down *Hg-pel-6* using *in vitro* RNA interference resulted in a 46.9% reduction of the number of nematodes that invaded the plants and a 61.5% suppression of the development of *H. glycines* females within roots compared to the GFP-dsRNA control. Plant host-derived RNAi induced the silencing of the *Hg-pel-6* gene, which significantly reduced the nematode infection levels at 7 Days post inoculation (dpi). Similarly, this procedure reduced the number of female adults at 40 dpi, which suggests the important roles of this gene in the early stages of parasitism. Our combined data suggest that two types of pectate lyases are present in the *H. glycines* genome and may have different roles during infection.
Introduction

Heterodera glycines Ichinohe, the soybean cyst nematode, is an obligatory endo-parasitic pathogen that is consistently the most damaging pest of soybean plants [1,2]. Recent estimates of the annual production losses caused by H. glycines range from $460 to $818 million in the US alone [3]. In China, the annual economic losses associated with H. glycines have been estimated to be $120 million [4]. Infective second-stage juveniles (J2) of H. glycines penetrate the soybean root tips and migrate intracellularly to the vascular cylinder to establish a permanent feeding site (syncytium) for its subsequent sedentary stages [5]. For plant parasitic nematodes (PPNs), the cell wall, which is primarily composed of pectin and cellulose, represents a formidable barrier to penetration and migration [6–7]. The synergistic effect of several enzymes is necessary for the degradation of pectin. These enzymes can be divided into the following two main groups: pectin esterases, which remove the methoxyl groups from pectin, and depolymerases (hydrolases and lyases), which cleave the back bones among galacturonate units [8]. For pectin degradation, two types of depolymerase, which include pectate lyase and polygalacturonase, have been isolated and characterized in PPNs. However, the pectin esterases that act on methylated pectin have not yet been recorded from PPNs [6].

The first pectate lyases from animal were discovered in the potato cyst nematode, Globodera rostochiensis [8]. Since then, pectate lyases have been isolated from several sedentary PPNs, such as species of Heterodera, Globodera, and Meloidogyne [9–13], and from migratory phytoparasitic nematodes, such as Bursaphelenchus xylophilus [14] and Aphelenchus avenae [15]. Subsequently, 15, 30 and 22 putative pectate lyases were predicted from the genome sequences of B. xylophilus [16], M. incognita [17] and M. hapla [18], respectively. These proteins are secreted by a pair of gland cells and are released into the plant tissue through the stylet of the nematode [19]. Pectate lyases, combined with a cocktail of cell wall-modifying enzymes (e.g., cellulases and hemicellulases), are thought to soften and degrade the structure of plant cell walls during nematode migration [6]. One of the two pectate lyases present in H. schachtii was silenced by RNAi, which resulted in fewer infections [13]. Moreover, the transient expression of Gr-pel2 in Nicotiana benthamiana leaves resulted in severe malformations of the infiltrated tissues at six dpi [12], indicating that pectate lyases play an essential role in nematode-plant interactions. However, an RNAi of hg-pel-2 in H. glycines caused a change in the sexual fate of the nematodes, which favored the male development but did not suppress the number of parasites in the root [20].

Here, four novel genes that encode pectate lyases were isolated from the soybean cyst nematode H. glycines. Furthermore, we analyzed the gene structure, expression characterizations and functional role of these novel pectate lyases.

Results

Identification of pectate lyases

Four ESTs similar to the pectate lyase of H. schachtii were identified in an EST dataset of H. glycines. Based on these sequences, four full-length cDNAs were obtained by the rapid amplification of cDNA ends (RACE). The sequence information of the four novel genes is shown in Table 1.

The respective lengths of the genomic sequences of Hg-pel-3, Hg-pel-4, Hg-pel-6 and Hg-pel-7 were 1,301 bp, 1,326 bp, 1,299 bp and 1,742 bp from the ATG to the stop codon. Comparisons of cDNA and genomic sequences showed that a single intron was present in Hg-pel-3, Hg-pel-4 and Hg-pel-6; in contrast, Hg-pel-7 contained 6 introns. The intron position and phase of Hg-pel-3, Hg-pel-4 and Hg-pel-6 were the same as those in Aa-pel-2 (GenBank No. AB495307),
Bx-pel-1 (GenBank No. AB232908), Bx-pel-2 (GenBank No. AB232909), Bm-pel-1 (GenBank No. AB232911) and Bm-pel-2 (GenBank No. AB232912); however, this position was not shared in Hg-pel-7. In addition, Gr-pel-1 (GenBank No. AF134582) from *G. rostochiensis* had six introns, and all of the intron positions and intron phase were fully shared with Hg-pel-6. Finally, Mi-pel-2 (GenBank No. AY515703) from *M. incognita* had two introns; the positions of these introns (but not the intron phase) were shared with two of the six introns of Hg-pel-6 and Gr-pel-1 (Fig 1).

**Sequence and phylogenetic analysis**

The sequence analysis indicated that the deduced protein sequences of Hg-PEL-3, HG-PEL-4 and HG-PEL-6 shared over 91% similarity and had a best BLAST hit with HS-PEL-2 (ABN14272) of *H. schachtii*. These sequences shared only 25.09% and 26.20% similarity with HG-PEL-1 and HG-PEL-2 of *H. glycines*, respectively; these latter proteins were previously described by Boer et al. (2002) and Gao et al. (2003), respectively. The similarities among HG-PEL-7, Hg-PEL-3, HG-PEL-4 and HG-PEL-6 were only in the range of 25.46%-26.20%; however, HG-PEL-7 was more similar to HG-PEL-1 and HG-PEL-2 with 94.46% and 95.20% of similarity, respectively. The identity and similarity values of pectate lyases among different cyst nematodes are shown in S1 Table. All of the deduced proteins had a predicted N-terminal signal peptide for secretion. In addition, the cleavage site of Hg-PEL-3, Hg-PEL-4 and Hg-PEL-6 was predicted between Gly20 and Gln21, whereas that of Hg-PEL-7 was predicted between Ala19 and Gln20. A conserved domain search confirmed that four conserved peptides of the PL3 family at the C terminal were identified from all the novel putative pectate lyase proteins (Fig 1). This search suggests that the sequences described here can be classified as new members of the PL3 family.

A phylogenetic analysis suggested that the seven putative pectate lyases of *H. glycines* were classified into two different branches (Fig 2). For instance, HG-PEL-3, HG-PEL-4, HG-PEL-5 and HG-PEL-6 were clustered with HS-PEL-2 of *H. schachtii*, HA-PEL-2 of *H. avenae* and pectate lyases of *Globodera* spp., *A. avenae* and *Bursaphelenchus* spp. In contrast, HG-PEL-7, Hg-PEL-1 and Hg-PEL-2 clustered together with HS-PEL-1 of *H. schachtii*, HA-PEL-1 of *H. avenae* and two pectate lyases of *G. mexicana*. However, other nematode sequences were not monophyletic; instead, they were clustered into three distinct clades.

**Southern blotting**

A Southern blot that contained genomic DNA from *H. glycines* was hybridized with a DNA probe designed to specifically hybridize with hg-pel-6 (Fig 3). Digestion with three different enzymes suggested that several related pectate lyase genes are present in the genome of *H. glycines*.  

| Gene name | GenBank accession | Full length cDNA excluding the poly (dA) tail (bp) | Putative ORF (bp) | Predicted protein (amino acids) | Molecular weight (kDa) |
|-----------|-------------------|-----------------------------------------------|-----------------|-------------------------------|-----------------------|
| Hg-pel-3  | HQ123255          | 926                                           | 765             | 254                           | 29.99                 |
| Hg-pel-4  | HQ123256          | 934                                           | 768             | 255                           | 29.72                 |
| Hg-pel-6  | HQ123258          | 929                                           | 762             | 253                           | 29.86                 |
| Hg-pel-7  | HQ123259          | 1,007                                         | 792             | 263                           | 27.96                 |

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Fig 1. Alignment of the putative protein sequences of the pectate lyases that belong to the polysaccharide lyase family 3. HG-PEL-1 [AAK08974], HG-PEL-2 [AAM74954] and HG-PEL-5 [ADW77534] correspond to *Heterodera glycines*; HS-PEL-1 [ABN14273] and HS-PEL-2 [ABN14272] correspond to *H. schachtii*; GR-PEL-1 [AAF80747] and GR-PEL-2 [AAM21970] correspond to *Golobedera rostochiensis*; GP-PEL-2 [ACU64826] corresponds to *G. pallida*; BX-PEL-1 [BAE48369] and BX-PEL-2 [BAE48370] correspond to *Bursaphelenchus xylophilus*; BM-PEL-1 [BAE48373] and BM-PEL-2 [BAE48375] correspond to *B. mucronatus*; AA-PEL-1 [BAI44999] and AA-PEL-2 [BAI44997] correspond to *Aphelenchus avenae*; MI-PEL-1 [AAQ09004] and MI-PEL-2 [AAQ97032] correspond to *Meloidogyne incognita*. Identical residues are highlighted in black. Black bars (I to IV) indicate the conserved regions.
In situ hybridization

The localization of Hg-pel-3, Hg-pel-4, Hg-pel-6 and Hg-pel-7 was analyzed in H. glycines by in situ mRNA hybridization (Fig 4). The result show that four transcripts of pectate lyase genes accumulated specifically within a pair of subventral gland cells by antisense cDNA probes. No signal was detected when the control sense cDNA probes were used (Fig 4).

Developmental expression analysis

The expression levels of Hg-pel-3, Hg-pel-4, Hg-pel-6 and Hg-pel-7 were compared at six developmental stages, i.e., pre-parasitic J2s, parasitic J2, J3, J4, adult females and eggs, using semi-quantitative RT-PCR (Fig 5). Hg-pel-3, Hg-pel-4 and Hg-pel-7 of H. glycines were expressed at high levels in pre-parasitic J2s and eggs. Moreover, Hg-pel-6 was expressed in eggs, pre-parasitic J2 and parasitic J2 and declined during further development of the nematode. In contrast, the expressions of the control gene (actin) were not different in different developmental stages (Fig 5).

In vitro RNAi

To determine the functional importance of pectate lyases during a nematode infection, Hg-pel-6 was knocked down using in vitro RNAi. The uptake of materials from a solution was induced by exposure to octopamine, as confirmed by the presence of a fluorescent signal in the stylet and median bulb of H. glycines at 20 h after soaking in a buffer that contained fluorescein isothiocyanate (FITC; Fig 6A). A semi-quantitative RT-PCR analysis showed that the Hg-pel-6 transcripts were not significantly reduced at 12 h after soaking; instead, the transcripts were increasingly suppressed at 16 h and 20 h (Fig 6B). Invasion studies showed that the treatment of J2 with hg-pel-6 dsRNA significantly reduced (46.9%, P = 0.001) the ability of the nematodes to infect plant roots compared to nematodes that were soaked in GFP dsRNA as a control. The number of adult females established within plants decreased by 61.5% at 30 Days post inoculation (dpi, Fig 6C). There were no significant differences between GFP dsRNA-treated controls and nematodes that were not exposed to dsRNA.

In planta RNAi

To confirm the results of the function analysis with in vitro RNAi, a representative Hg-pel-6 cDNA sequence was used to express dsRNA in planta driven by the CaMV35S promoter. The hairpin constructs were confirmed by the expression of the dsRNA using RT-PCR (Fig 7A). The Hg-pel-6 transcript level significantly declined after the treatment of in vivo RNAi (Fig 7B). The transgenic expression of the Hg-pel-6 hairpin construct resulted in a 30.4–39.1% reduction in the number of nematodes compared to the GFP control at 7 dpi (Fig 7C). Furthermore, the number of adult females on the hairy roots decreased by 36.4–45.5% at 40 dpi (Fig 7D), which suggests an important role of HG-PEL-6 during parasitism.

Discussion

In this article, we report the identification and functional analysis of four novel pectate lyase genes from H. glycines. The sequence similarity between HG-PEL-3, HG-PEL-4, HG-PEL-6,
Fig 2. Phylogenetic tree of the polysaccharide lyase family 3 generated using maximum likelihood of PhyML. The numbers next to the branches indicate the bootstrap value. GenBank accession numbers of pectate lyase proteins correspond to *Meloidogyne incognita* (MI-PEL-1, MI-PEL-2 and MI-PEL-3), *M. hapha* (Mh-ENG-1 and Mh-ENG-2), *M. javanica* (MJ-PEL-1), *Globodera rostochiensis* (GR-PEL-1 and GR-PEL-2), *G. pallida* (Gp-ENG-1 and Gp-ENG-3).
HG-PEL-7 and the published pectate lyases, i.e., HG-PEL-1 and HG-PEL-2, was relatively low, but all of the sequences contained conserved regions [21] and characteristic cysteines [8] of PL3 pectate lyases (Fig 1). Therefore, we considered them to be members of the PL3 pectate lyase family, which also includes all of the pectate lyases identified from other plant-parasitic nematodes to date [8–15].

Previous research showed that there were at least two types of pectate lyases in *H. schachtii* [20] and *G. rostochiensis* [12]; however, only one type was found in *H. glycines*. In this study, three of the four described sequences were significantly different from the previously cloned HG-PEL-1 and HG-PEL-2. This outcome indicates that the two types of pectate lyases were also present in genome of *H. glycines* and that they may play difference roles. Herron et al. [22] suggested that a pathogen that secretes a battery of pectinolytic enzymes would be expected to have a broad host range because each of these enzymes recognizes a different sequence of methylated and non-methylated oligo galacturonate units. In addition, our phylogenetic study revealed that the pectate lyase proteins of *H. glycines* were clustered into distinct clades. This result, combined with the distinct patterns of intron number and position between the two types of sequences, suggests that the pectate lyase genes of *H. glycines* may have evolved from at least two ancestral genes. These results are consistent with previous research, which suggests that the PL3 of plant parasitic nematodes was acquired by a few independent lateral gene transfers (LGT) events from different subfamilies [23].

The *in situ* hybridization experiments localized the mRNA of *Hg-pel-3, Hg-pel-4, Hg-pel-6* and *Hg-pel-7* in the subventral esophageal glands. These results are consistent with data from other phytopathogenic nematodes [9–15] and further confirm that the subventral gland cells of endo-parasitic nematodes function during the early parasitic process of plant invasion and migration of J2s through plant tissues [24]. The gland-cell-specific expression location together with the presence of predicted signal peptides on all the putative proteins suggest that pectate lyases possible secretion may play a key role during nematode infection. Three of the four novel pectate lyase genes are highly expressed in pre-parasitic J2s and eggs, which suggests that these genes contribute to pectin degradation and assist in the root invasion and successive migration of the nematode through the plant roots. The developmental expression pattern is similar to that previously observed in *H. schachtii* and *M. incognita* [11,13]. However, *Hg-pel-6* is also expressed during the parasitic stages, which suggests that this protein may play a key role in the latter stages of the parasitic interaction. A similar result was described for *Mi-pel-1* in *M. incognita* [11].

Two pectate lyases, *Hg-pel-1* and *Hg-pel-2*, were previously isolated and characterized from *H. glycines* [9]. Because *Hg-pel-6* and these proteins shared low similarity and because *Hg-pel-6* had a different developmental expression pattern compared to the other sequences, this gene was selected and knocked down by RNAi to investigate its functional role. RT-PCR showed that the silencing of *hgl-pel-6* occurred 16 h after soaking and increased 20 h after soaking. These results confirm those of Chen et al. [25], who described that silencing was induced in *G. rostochiensis* after it was soaked for 24 h. In general, the RNAi phenotype will be enhanced with an increase in the soaking time, particularly for cyst nematodes [26]; however, the RNAi of other genes can also be carried out with 4 h of incubation in the cyst nematode [27,28].

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Fig 3. Southern blotting of Hg-pel-6. Southern blot of the genomic DNA from *H. glycines* digested with *EcoRI, EcoRV* and *HindIII* as well as hybridized with an *hg-pel-6* cDNA probe. The DNA size markers are indicated in kb.

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Bakhetia et al. [20] reported that the silencing of hg-pel-2 induced a change in the sexual fate that favored the male development but did not suppress the number of infections. Our results demonstrate that a reduction in the Hg-pel-6 mRNA resulted in lower infections. Similar results have been reported in *H. Schachtii* [13]. Furthermore, the development of adult females *H. Glycines* was also suppressed. These results imply that Hg-pel-6 plays an important role in the infection process. The low sequence similarity observed between Hg-pel-6 and Hg-pel-2 and the fact that knocking down Hg-pel-6 and Hg-pel-2 by RNAi induced different phenotypes indicate that the two types of pectate lyases in the soybean cyst nematode may play different roles during infection.

Our study shows that *H. Glycines* targets pectic polysaccharides with at least two types of pectate lyase genes. Moreover, these genes may perform separate functions in different developmental stages. These novel putative pectate lyases of *H. Glycines* are important in the infection process. These experimental results agree with the general idea that PPNs secrete a diverse range of enzymes to loosen and degrade the plant cell wall structure, thus facilitating the migration through the root tissue [8].

**Materials and Methods**

**Nematode culture**

*H. Glycines* race 4 was isolated from Langfang City, Hebei Province, China. A single cyst was cultured on soybean (*Glycine max*) roots in autoclaved soil and grown in a greenhouse. Eggs were collected from *H. Glycines* cysts, and hatched pre-parasitic J2 were collected by placing the eggs on a sieve (25 μm pore size) in 3 mM ZnSO₄ at 25°C. To isolate the parasitic juveniles and later life stages of *H. Glycines*, 10-day-old soybean plants were inoculated with *H. Glycines* J2
Infected roots were harvested at 4, 8, 14 and 30 dpi to collect parasitic J2s, J3s, J4s and adult females, respectively. These stages were isolated as previously described [29].

**DNA and RNA isolation and cDNA synthesis**

Genomic DNA was isolated from approximately 5000 pre-parasitic J2s with a DNeasy Blood & Tissue Kit (Qiagen, GmbH, Germany). Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). mRNA was isolated from pre-parasitic J2s, parasitic J2s, J3s, J4s, adult females and eggs with a Dynabeads mRNA Direct kit (Invitrogen, Carlsbad, CA, USA). The first-stranded cDNA was synthesized with SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**Isolation of pectate lyases**

Specific primers were designed to amplify the 3’ and 5’ cDNA ends of four sequences identified from *H. glycines* expressed sequence tags (ESTs) in the GenBank. To obtain full length cDNAs, the 5’ and 3’ cDNA ends of the pectate lyases were amplified from total RNA of pre-parasitic J2 with specific primers (Table 2) using a GeneRacer kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The genomic sequences of the pectate lyases were
Fig 6. RNAi uptake of *Heterodera glycines* and infections of soybean roots with nematodes following the *Hg-pel-6* dsRNA treatment. The fluorescein isothiocyanate (FITC) uptake was observed by fluorescence microscopy (A), and the activity of the nematodes after soaking was observed under a light microscope (B). S, stylet; M, median bulb; pl, pharyngeal lumen. Scale bar: 50 μm. (C) An RT-PCR analysis for the expressions of *Hg-pel-6* and *actin*.
obtained from genomic DNA with PEL3-FF, PEL3-FR, PEL4-FF, PEL4-FR, PEL6-FF, PEL6-FR, PEL7-FF and PEL7-FR. All amplicons were cloned into PMD-18 vector (TaKaRa Biotech, Dalian, China) and sequenced by the Beijing Genomics Institute.

Fig 7. Effect of plant host-derived RNA-interference of \( Hg\text{-}pel\text{-}6 \) on the hairy roots of soybean plants by \( H. \) \( glycines \).

(A) An RT-PCR of the single-stranded PDK intron of the hairpin dsRNA was used to confirm the expression of the \( hg\text{-}pel\text{-}6\text{-}dsRNA \) and non-specific control GFP dsRNA (RNAi) in independent hairy roots of soybean lines at 10 d post-germination. (B) A semi-quantitative RT-PCR confirmed the knockdown of \( Hg\text{-}pel\text{-}6 \) expression after an RNAi treatment of \( H. \) \( glycines \). (C and D) Number of \( H. \) \( glycines \) per root system that expressed \( Hg\text{-}pel\text{-}6\text{dsRNA} \) at 7dpi and 40dp. The error bars indicate the standard error of the mean; \( n = \text{8} \). The asterisk represents a significant difference (t test, \( P < 0.05 \)) with respect to the control.

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Sequences analysis

A total of 24444 ESTs from *H. glycines* were downloaded from the GenBank, clustered and assembled using CAP3 [30]. BLAST analyses were performed on the non-redundant (nr) databases at the NCBI to identify the sequence similarity [31]. The ORFs of pectate lyases were analyzed using the ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptides
of proteins were predicted using SignalP 4.0 [32]; the transmembrane domain prediction was performed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). The molecular weight (Mw) was analyzed using Compute pI/MW tool (http://web.expasy.org/compute_pi/ website). The multiple sequence alignment was performed with ClusterW. The conserved domain searches were carried out with the mature protein sequences at the NCBI and InterProScan (www.ebi.ac.uk/InterProScan/).

**Phylogenetic Analysis**

The published pectate lyase sequences were downloaded from the GenBank. The amino acid sequences without signal peptides were aligned using Muscle, alignment curation was performed with Gblocks (v0.91b). The phylogeny was reconstructed from the alignment on the Phylogeny.fr platform [34] based on the maximum likelihood method (PhyML program) and reliability for internal branches was assessed using the bootstrapping method (500). A default substitution model was selected assuming an estimated proportion of invariant sites (of 0.021) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The tree rendering was performed using TreeDyn software (v1.83) and MAGE 5.0 [35].

**Southern hybridization**

The genomic DNA of *H. glycines* was digested with EcoRI, EcoRV and HindIII (New England Biolabs, Ipswich, MA, USA). The resulting fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to a positively charged Nylon Membrane (GE Healthcare Biosciences, NJ, USA). A cDNA probe was synthesized using a PCR DIG Probe Synthesis Kit (Roche Molecular Diagnostics, Mannheim, Germany) with specific primers (PEL6-TZF and PEL6-TZR; Table 2). Pre-hybridization (in DIG Easy Hyb at 42°C for 2 h), hybridization (in DIG Easy Hyb at 42°C overnight) and immunodetection were performed following the manufacturer’s instructions using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Molecular Diagnostics, Mannheim, Germany).

**In situ hybridization**

An in situ hybridization was carried out according to de Boer *et al.* [36] and Long *et al.* [37] with minor modifications. The sense and anti-sense probes were generated by asymmetric amplification and labeled using digoxigenin-11-dUTP (Roche Molecular Diagnostics, Mannheim, Germany) with gene specific primers (PEL3-TZ-F, PEL3-TZ-R, PEL4-TZ-F, PEL4-TZ-R, PEL6-TZ-F, PEL6-TZ-R, PEL7-TZ-F and PEL7-TZ-R; Table 2). Freshly hatched J2 nematodes were washed three times with 1× phosphate-buffered saline (PBS) buffer (Sigma-Aldrich, St. Louis, MO, USA); then, they were fixed overnight in 4% paraformaldehyde at room temperature and cut into two to five pieces. The hybridization was carried out overnight at 50°C. Hybridized probes were detected with a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Molecular Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The nematodes were examined using microscopy (Leica, DM2500, Germany).

**RT-PCR**

Fragments of pectate lyases from six developmental stages of *H. glycines* were PCR-amplified for 30 cycles with the primers used in the above in situ hybridizations (Table 2). Actin (AF318603) was amplified as a positive control using the primers ACT-F and ACT-R.
The gene silencing effect was tested with an RT-PCR after soaking in dsRNA with the primers PEL6-TZ-F and PEL6-TZ-F (Table 2). The PCR products were separated on a 1.5% agarose gel and stained with Ethidium Bromide (EB).

In vitro RNA interference

The RNAi method was based on that described by Urwin et al. [27] and Sukno et al. [38] with minor modifications. Briefly, the dsRNA was designed within the coding region of Hg-pel-6 from nucleotides (298–595 bp), synthesized and purified with a MEGAscript RNAi Kit (Applied Bio-systems, Austin, TX, USA) following the manufacturer’s instructions using the primers PEL6-T7-F and PEL6-T7-R (Table 2). A dsRNA against GFP was prepared from a gfp-containing construct using primers GFP-T7-F and GFP-T7-R. The dsRNA was re-suspended in DEPC-treated water and quantified by spectrophotometry. Approximately 3000 freshly hatched J2 were soaked in 1/4×M9 buffer (43.6 mM Na2HPO4, 22 mM KH2PO4, 2.1 mM NaCl, 4.7 mM NH4Cl) that included 50 mM octopamine, 3 mM spermidine, 0.05% gelatin, and 2 mg/ml dsRNA at room temperature, in the dark on a rotator. Control samples were incubated in the same solution but without dsRNA or with dsRNA targeted against GFP. Approximately 300 J2 individuals were moved from the soaking buffer to perform RT-PCR at 4, 8, 12, 16 and 20 h after soaking. Those nematodes were washed three times in ¼ ×M9 to remove the dsRNA and stored at -80°C before being used for RNA extractions. The remaining J2s (c. 1,800) were used for infection tests as described below. All experiments were performed in triplicate.

Susceptible Glycine max (soybean) cv. Lee68 was sown in 8 cm pots with autoclaved soil. Approximately 300 treated nematodes were inoculated into the soybean roots as described previously [20] and cultured at 25°C. After 4 days, the soybean roots from three pots were collected and stained using acid fuchsin, as described by Bird et al. [39]. Then, the infected nematodes within the roots were counted under the microscope. The remaining soybean roots were harvested at 30 dpi, and the numbers of adult female were counted under the microscope. All experiments were performed in triplicate, and the results were analyzed with the SAS software (V8.3) using Duncan’s multiple range test.

In planta RNA interference

Two inverted repeat sequences of Hg-pel-6 cDNA which was used to synthesized the dsRNA in vitro RNAi was amplified and inserted into the pHANNIBAL (RNAi) vector [40] to express the hairpin dsRNA of Hg-pel-6 in a transformed hairy roots of soybean with primers pel6-IR-F and pel6-IR-R (Table 2). A fragment of green fluorescent protein (GFP) cDNA sequence was also constructed in pHANNIBAL with primers GFP-IR-F and GFP-IR-R (Table 2) in this study as a negative control. The Not I fragments, containing a repeat sequence from the pHANNIBAL vector, were sub-cloned into the binary vector pART27 [41]. The constructs were transformed into Glycine max (soybean) cv. Williams 82 with Agrobacterium rhizogenes strain K599 as described previously [42]. The dsRNA hairpin construct in hairy roots of soybean was confirmed by RT-PCR using a specific primer set, PDK-R and PDK-R (Table 2). The positive root lines were sub-cultured and used for further inoculation.

H. glycines J2s were hatched and surface-sterilized in 0.1% chlorhexidine digluconate (Sigma-Aldrich, St. Louis, USA) and 0.5 mg ml⁻¹ hexadecyltrimethylammonium bromide (CTAB, Sigma-Aldrich, St. Louis, USA) for 20 min, followed by three washes with sterile distilled water as described previously [43], then suspended in 1.5% low melting point agarose (Invitrogen, Carlsbad, CA, USA). The active nematodes were counted under a microscope after the suspension. Approximately 30 active nematodes were inoculated onto each infection
point, and three inoculation points were used per root plate. Twenty plates were inoculated per line, with three independent lines for Hg-pel-6, one line for GFP and an empty vector control. Eight plates were stained at 7 dpi, and female adults on the surface of roots from same plates at 40 dpi were counted as described above. All the results were analyzed with the methods described above. This procedure was performed to obtain H. glycines feeding on RNAi over-expression plants. The transcript levels of hg-pel-6 in the feeding stage of H. glycines were tested with an RT-PCR using the primer set PEL6-TZ-F and PEL6-TZ-R. An actin fragment of H. glycines was also used as a positive control using the primers ACT-F and ACT-R (Table 2).

Supporting Information

S1 Table. Percentages of protein similarities and identities (bold) of pectate lyases among the different cyst nematodes.

(DOC)

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Author Contributions

Conceived and designed the experiments: DLP XQH. Performed the experiments: HP JKC WKH HBL WTH. Analyzed the data: HP. Contributed reagents/materials/analysis tools: SML LAK HBL. Wrote the paper: HP JKC.

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