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Differential expression of *Rs-eng-1b* in two populations of *Radopholus similis* (Tylenchida: Pratylecnchidae) and its relationship to pathogenicity

Chao Zhang · Hui Xie · Chun-Ling Xu · Xi Cheng · Ke-Mei Li · Yu Li

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Abstract We constructed a SSH (suppression subtractive hybridization) library based on two populations (*Rs*-C and *Rs*-P) of *Radopholus similis* from different host plants and exhibiting differences in pathogenicity on *Musa paradisiaca* and *Anthurium andraeanum* plants. In order to screen the clones with significant expression differences from the SSH library, a total of 2,400 clones was randomly selected and reverse northern blotting was performed on them. Out of the 2,400 clones, 89 clones showed significant expression differences. Out of sequencing these 89 clones, distinct sequences from 87 clones were obtained. Aligning the 87 distinct sequences against the non-redundant nucleotide database (nr) in NCBI, we found that five sequences were highly conserved with *Rs-eng-1b*. Two of five sequences with lengths of 467 base pairs (bp) (GW395922) and 742 bp (GW395923) were further employed to perform 5′ RACE-PCR and 3′ RACE-PCR, respectively. Subsequently, the complete length of *Rs-eng-1b* (EU414839) was obtained (1,427 bp). Our qPCR result showed that expression of *Rs-eng-1b* in the population *Rs*-C with high pathogenicity on host plants was approximately 2.7 times as much as the expression of *Rs-eng-1b* in the population *Rs*-P with low pathogenicity on host plants. Furthermore, the gene *Rs-eng-1b* from the *Rs*-C population also showed expression differences amongst four different development stages. The order of *Rs-eng-1b* relative expression abundance from high to low was females, juveniles, males, and eggs. We further used RNAi to test whether *Rs-eng-1b* of *Rs*-C population was responsible for pathogenicity which was the first RNAi work about *Rs-eng-1b*. The RNAi results showed that *Rs-eng-1b* expression had a positive correlation to pathogenicity of the population. The longer the RNAi treatment, the less pathogenic the nematode population was. Non-endogenous *gfp* dsRNA had no significant influence on the expression of *Rs-eng-1b* and pathogenicity of *R. similis Rs*-C population. In conclusion, all our evidence indicated *Rs-eng-1b* might be a crucial pathogenicity-related gene in *R. similis*.

Keywords Banana burrowing nematode · SSH · β-1 · 4-endoglucanase · Real-time PCR · RNAi

C. Zhang · H. Xie (✉) · C.-L. Xu · X. Cheng · K.-M. Li · Y. Li
Laboratory of Plant Nematology and Research Center of Nematodes of Plant Quarantine, College of Natural Resource and Environment, South China Agricultural University,
Guangzhou 510642, People's Republic of China
e-mail: xiehui@scau.edu.cn

Introduction

The banana burrowing nematode, *Radopholus similis* is one of the most damaging pests of bananas and severely harms pepper, ornamental plants, and many other agronomic and horticultural crops. *R. similis* is

known to attack over 250 plant species (O'Bannon 1977). *R. similis* is a migratory endoparasite that invades the roots and feeds on the cytoplasm of cortex cells. As a result, the roots will blacken and die which results in a reduction of plant growth and development. This destruction of crops leads to severe economic losses and consequently, many countries have entered it on the list of quarantine plant pests (Cotton and Van Riel 1993; Smith and Charles 1998).

Nematicides have been used as one of many integrated approaches to control plant-parasitic nematodes (PPNs). As concerns have arisen over the environmental implications associated with over-using of some nematicides, many nematicides have been decreased in use. As a result, shortcomings in our ability to successfully limit yield loss have occurred. Thus, an effort to seek additional approaches for PPNs control is imperative.

In recent years, molecular biology and genetic engineering approaches to solve the problem of PPNs control have been developed quickly. In order to have more appropriate biological controls, understanding the molecular mechanisms involved in pathogenicity by mining pathogenicity genes has become extremely important (Chen et al. 2005). It is an effective method to screen for pathogenicity genes by comparing gene expression profiles between different populations of a species exhibiting different levels of pathogenicity on host species. There are a series of approaches to identify differentially expressed genes, such as mRNA differential display reverse transcription PCR, representational difference analysis (RDA), suppression subtractive hybridization (SSH), and reciprocal subtraction differential RNA display (RSDD). Of those approaches, SSH has been extensively applied in molecular genetics and molecular biology (Kuang and Ashorn 1993; Tchernitsa et al. 1999; Shen and Liu 2004).

SSH has super sensitivity to recognize the differentially expressed genes in low expression abundance (Diatchenko et al. 1999). SSH advantages include high sensitivity, low occurrence of false positives, high efficiency, and low cost. Commonly, SSH is better than other approaches to detect differentially expressed genes, since more than 100 differentially expressed genes can be enriched in one SSH (von Stein et al. 1997). Grenier et al. (2002) used SSH to investigate differentially expressed genes between two different populations of *Globodera pallida* and identified a cellulase and an important pathogenicity factor. Meanwhile, several parasite-related genes from *Meloidogyne incognita* were detected by SSH (Huang et al. 2004). Although mining differentially expressed genes in PPNs is not very difficult, it is crucial to understand gene function involved in pathogenesis and regulation patterns. RNA interference (RNAi) is an RNA-dependent gene silencing process in which the guide strand (small interference RNA, siRNA) from short double-stranded RNA (dsRNA) molecules are incorporated into the RNA-induced silencing complex (RISC) to bind to specific mRNA molecules (target mRNA). siRNAs prevent target mRNAs from being translated into protein by either degrading target mRNAs or inhibiting ribosomes from completely translating the mRNA. RNAi has been found in many eukaryotes including animals and plants. RNAi was initially discovered and developed to application on potato (Kawchuk et al. 1991) and *Caenorhabditus elegans* (Guo and Kemphues 1995; Fire et al. 1998). RNAi can be used as a simple and effective alternative gene-knockout tool to obtain function-less or function-loss mutants, because of its high sequence-specificity and effective interference activity. Meanwhile, with its simple operation, short cycle and low cost, it has become an extremely important tool and most popular research topic in the fields of gene identification, genetic analysis, gene function, gene therapy and genomics (Barstead 2001; Kamath et al. 2000; Maine 2001; Cheng et al. 2003; Rangasamy et al. 2004; Tijsterman et al. 2004). Although it is difficult to study gene function of PPNs by constructing mutants because PPNs are obligatory parasites and do not grow in artificial culture medium; with the advantage above, RNAi could be used for screening mutants, identifying gene function, or control of PPNs (Bakhetia et al. 2007). However, little research has been carried out on the pathogenicity genes of PPNs, and their functions; and, so far, research has also concentrated on the sedentary endoparasitism nematodes, such as *Meloidogyne* sp., *Heterodera* sp. and *Globodera* sp. (Goddijn et al. 1993; Brindley et al. 1997; Vercauteren et al. 2002; Shingles et al. 2007). The molecular mechanism of parasitic-related-gene-mediated infection in the plant host-parasite interaction is still unclear.

β-1, 4-endoglucanase (EGases), also known as cellulase, functions in degrading plant cellulose and is classified as a fifth glycosyl hydrolase family

(Haegeman et al. 2008). EGases was first thought to originate through horizontal gene transfer (HGT) (Jones et al. 2005). Chen et al. (2005) and Bakhetia et al. (2007) employed RNAi to silence EGases in *Globodera rostochiensis* and *Heterodera glycines*, respectively. Their investigation showed that the nematodes with silenced EGases had significantly less pathogenicity on their host plants. It has been widely accepted that EGases plays a key role in pathogenicity in the two nematodes. Therefore, EGases is viewed as a prospective target gene that could be developed to provide resistance to the nematodes. The first high throughput molecular characterization of *R. similis* was studied by Jacob et al. (2008), and much useful information was discovered; some genes involved in parasitism, including EGases were identified. The four EGases genes in *R. similis* had been cloned by Haegeman et al. (2008). Their research only focused on tissue-expression and time-expression (Haegeman et al. 2008). Currently, there is no report on whether the function of EGases is involved in pathogenicity of *R. similis* on plant.

In this study, a differential gene expression library was constructed by SSH, based on two populations of *R. similis* from different areas and different hosts. EGases in *R. similis* (*Rs-eng-1b)* was selectively screened from a SSH library. In order to clarify the gene function, a series of experiments including RNAi, real-time PCR (qPCR), carrot callus culture, and artificial infection, were performed to investigate the relationship between the expression of *Rs-eng-1b* and pathogenicity of *R. similis* on plant.

Materials and methods

Plant materials

Anthurium, *Anthurium andraeanum* plants were bought from the Flowers and Plants Research Center, Guangzhou, Guangdong. The roots of anthurium seedlings were washed with sterile water and nematodes collected on nested 0.147 mm and 0.026 mm pore sieves. Baermann funnels (Viglierchio and Schmitt 1983) were used to separate nematodes from the precipitation and microscope inspection was employed. If there were nematodes in roots, the roots were treated to remove nematodes (Tsang et al. 2004). All non-contaminated seedlings were grown in sterilized soil medium for 15 days for later use.

Nematodes

Two populations of *R. similis*: *Rs*-C and *Rs*-P, which were collected from roots of ornamental plants *Calathea makoyana* and *Philodendron cv* Green Emerald, respectively, and the internal transcribed spacer (ITS) regions of *Rs*-C and *Rs*-P were sequenced. The different pathogenicity of the two nematode populations was certified on *Musa paradisiaca* by a member of our group (personal communication). These populations were cultured on excised carrot (*Daucus carota*) disks in Petri dishes with a diameter of 6 cm at 25 °C in incubator (Fallas and Sarah 1994).

Nematode extraction

The carrot callus was mashed with a blender. The mashed solution was filtered through combined sieves with aperture of 0.147 mm and 0.026 mm. Nematodes were collected from the 0.026 mm aperture sieve.

Roots of anthurium were cut into 1 cm fragments. These fragments were mashed in a blender and further filtered by nested sieves with apertures 0.147 mm and 0.026 mm. Nematodes were collected from the 0.026 mm aperture sieve. This first nematode collection was labelled as N1. Meanwhile, the 0.147 mm pore sieve subjected to the Baermann funnel separating the nematodes number was labelled as N2. The total nematodes in roots were calculated as the sum of N1 and N2. Nematodes in soil were isolated from 200 ml of mixed soil from the host plant pot using a Baermann funnel method. Extraction from sieve or Baermann funnel were adjusted to 10 ml of nematode suspension, and 1 ml of the suspension was pipetted into a glass dish with a diameter of 6 cm, and the number of nematodes were counted under the stereomicroscope, while the number of females, males and juveniles were counted respectively. The same work was done 10 times until all nematode suspensions had been counted. Total nematode population size was calculated as the sum of nematodes isolated from roots (N1 + N2) and soil.

RNA extraction

About 20,000 mixed-stage nematodes from each population separated from carrot disks were respectively collected in an Eppendorf tube and washed with diethypyrocarbonate (DEPC) water three times. Cleaned

nematodes were ground in liquid nitrogen. Total RNA was extracted from the nematodes using TRIZOL following manufacturer instructions (Invitrogen) and further treated with DNase I (Promega) for 15 min at 37 °C. The RNA was verified by 1.0 % agarose gel electrophoresis and was stored −80 °C for later use.

Only 500 mixed stages nematodes separated from carrot disks were treated by RNAi. Therefore, a MicroElute total RNA kit (OMEGA) was used to extract the total RNA according to the kit operation protocol.

SSH library

A SMARTer PCR cDNA Synthesis Kit (Clontech) was used to transcribe total RNAs from *Rs*-C and *Rs*-P nematode populations into cDNA according to the manufacturer's protocol. Resulting cDNAs underwent Suppression Subtractive Hybridization (SSH) using a PCR-Select cDNA subtraction kit (Clontech). The secondary PCR amplified product was cloned into the vector pGEM-T easy (Promega) and was then transformed into complete *Escherichia coli* JM109 cells. Finally, single clones were randomly selected for later PCR detecting and sequencing.

Reverse Northern blotting

Total RNAs from *Rs*-C and *Rs*-P nematode populations were transcribed into cDNA according to operation instruction of a ReverTra Ace qPCR RT kit (TOYOBO). To synthesize Probe-C and Probe-P from *Rs*-C and *Rs*-P nematode populations, a DIG high prime DNA labelling and detection starter kit I (Roche) was used to label the cDNA templates. The concentration of probes was quantified by a Nano-Drop spectrophotometer. According to operation protocol of DIG high prime DNA labelling and detection starter kit I (Roche), the PCR products from SSH were hybridized with Probe-C and Probe-P.

Sequencing and alignment analysis

After hybridization, the results of the hybridized signals were analysed by UV transilluminator (Alpha Innotech). Blots that had two-fold differences in reverse northern blotting signals were selected for sequencing. After removing vector sequences and adapter sequences, sequences were aligned against a non-redundant protein database (nr) and a non-redundant nucleotide database (nt) in NCBI.

Obtaining complete sequence of *Rs-eng-1b*

The fragment candidate of *Rs-eng-1b* was screened out of the library with alignment analysis. To obtain the complete sequence of *Rs-eng-1b*, 3′RACE primers (NEST-S1 and NEST-S2) (Table 1) and 5′ RACE primers (NEST-A1and NEST-A2) (Table 1) were designed to amplify the 3′ end and 5′ end of *Rs-eng-1b* using a SMART RACE cDNA amplification kit (Clontech), respectively. Finally, we spliced three fragments of *Rs-eng-1b* (5′ end, middle fragment, and 3′ end) into the complete sequence of *Rs-eng-1b*. Two specific primers (cds-F and cds-R) (Table 1) from spliced complete sequences of *Rs-eng-1b* were designed to form the complete sequence of *Rs-eng-1b*.

Expression analysis of *Rs-eng-1b* and qPCR

qPCR was used to assess the variation in the expression levels of *Rs-eng-1b* between *Rs*-C and *Rs*-P nematode populations, and among different development stages of *Rs*-C: females, males, juveniles and eggs. *Rs*-C and *Rs-P* were isolated from infected carrot callus for total RNA extraction.

One hundred females, males, juveniles and eggs respectively were used for RNA extraction. The RNA was then quantified and qualified using a Nano-drop spectrophotometer, and then stored at −80 °C for further analysis. All the RNA used for qPCR was prepared from three different samples as three biological replicates.

Based on the complete sequence of *Rs-eng-1b*, specific primers qPCR-F and qPCR-R (Table 1) were designed to represent *Rs-eng-1b* expression. According to the method described by Jacob et al. (2007), the primers Actin-F and Actin-R were synthesized (Table 1) to amplify the reference gene, β-actin. qPCRs were performed on mixed life stages of *Rs*-C and *Rs*-P and females, males, juveniles, and eggs of *Rs*-C, and reactions were performed in triplicate on CFX-96 (Bio-Rad) qPCR machine, using SYBR Green qPCR Master Mix-plus kit (TOYOBO) according to the manufacture's protocol with the following reaction conditions: 95 °C for 15 s and 60 °C for 30 s (40 cycles). Initial data analysis was carried out using the Bio-Rad CFX-96 manager software, which created Ct values and extrapolated relative levels of PCR products from

Table 1 Primers used for RACE of endoglucanase *Rs-eng-1b* gene sequence, quantitative polymerase chain reaction (qPCR) for *Rs-eng-1b* expression in *Rs*-C population and *Rs*-P population and different development stage of *Radopholus similis,* and to generate double-stranded (ds) RNA of *Rs-eng-1b* and non-endogenous *gfp* dsRNA control

Primer	Sequence	Source
Actin-F	5′-GAAAGAGGGCCGGAAGAG-3′	Jacob et al. (2007)
Actin-R	5′-AGATCGTCCGCGACATAAAG-3′	Jacob et al. (2007)
NEST S1	5′-ACGAGACCTACAATGAGC-3′	
NEST S2	5′-AGCCTGCCCGTGTTCGTGAC-3′	
NEST A1	5′-GGCAGGTACTCGGTCACG-3′	
NEST A2	5′-CATTGTAGGTCTCGTACC-3′	
qPCR-F	5′-AATCTCTTACGTGAACTGGGC-3′	
qPCR-R	5′-GGTCGCTCCAGATTTAGTCG-3′	
cds-F	5′-TCCGCTTTCACCGCTTTCA-3′	
cds-R	5′-CAGACATTCAGCATCCA-3′	
T7S	5′-TAATACGACTCACTATAGGGGCTGTT CTGGTCGCAATG-3′	
A	5′-CAGAGGTGGGCTCATTGTAG-3′	
T7A	5′-TAATACGACTCACTATAGGGCAG AGGTGGGCTCATTGTAG-3′	
S	5′-GCTGTTCTGGTCGCAATG-3′	
G-T7S	5′-GGATCCTAATACGACTCACTATAGGG CACAAGTTCAGCGTGTCCGGCG-3′	
G-A	5′-CGATGCGGTTCACCAGGGTGTCG-3′	
G-T7A	5′-GGATCCTAATACGACTCACTATAGGG CGATGCGGTTCACCAGGGTGTCG-3′	
G-S	5′-CACAAGTTCAGCGTGTCCGGCG-3′	

standard curves. Melt curves were done routinely, and this allowed the possibility of both contamination and primer dimers to be discounted. Actin was used as a positive control in all experiments. All experiments were performed in triplicate.

Synthesis of *Rs-eng-1b* dsRNA

A fragment of about 450 bp from the ORF of *Rs-eng-1b* was cloned into the vector PMD18-T (TAKARA). The constructed vector was further confirmed by sequencing. Based on the fragment, specific primers (T7S, A, T7A, and S) (Table 1) with a T7 promoter were designed to amplify the sense and anti-sense product. Sense and antisense RNA were transcribed using T7 transcription kit (TOYOBO) according to the manufacturer's instructions. Sense and antisense transcripts were annealed for 30 min at 37 °C and analyzed by agarose gel electrophoresis. The dsRNA was purified by equal amount of LiCl (3 mol/l) overnight and washed by 70 % ethylalcohol three times, finally stored at −80 °C for later use. Non-endogenous control dsRNA (125 bp) (green fouorescent protein gene, *gfp*) was generated with designed specific primers (G-T7S, G-A, G-T7A, and G-S) (Table 1) from the cloning vector PYL 322-d1-GFPn supplied by QL Zhu, College of Life-Science, South China Agricultural University.

Rs-eng-1b's RNAi treatment and silence detection

Five hundred nematodes from the *Rs*-C population cultivated on carrot callus were collected and transferred in an Eppendorf tube, treated with DEPC water, and soaked in 50 μl *Rs-eng-1b* dsRNA solution (2 μg/μl) at room temperature for 12 h, 24 h, 36 h and 48 h, respectively. Non-endogenous *gfp* dsRNA solution (2 μg/μl) was used as a control. The treatment times used for the control were same as *Rs-eng-1b* dsRNAs. Meanwhile, untreated nematodes were used as a blank control. Treated nematodes were cleaned with DEPC water three times and the total RNA was then extracted. qPCR was used to analyze transcript suppression of *Rs-eng-1b* in the nematodes after the RNAi treatments. All experiments were performed three times.

Reproduction and pathogenicity of nematodes

Thirty female *Rs*-C nematodes were treated with *Rs-eng-1b* dsRNA solution (2 μg/μl) for 12 h, 24 h and 36 h. Controls were treated with *gfp* dsRNA solution (2 μg/μl) for 12 h, 24 h and 36 h, or without any treatment with dsRNA solution. Another control group of thirty female *Rs*-P nematodes without treatment with dsRNA solution was also included. All nematodes were then inoculated onto a carrot callus which were then maintained in a dark incubator at 25 °C for 56 days after which total nematodes in the carrot callus were isolated and calculated as described above. Each treatment was repeated five times.

The pathogenicity of *Rs-eng-1b* RNAi treated nematodes, *gfp* dsRNA solution treated nematodes, and untreated nematodes were compared. Each treatment was adjusted to a suspension with 100 nematodes per ml. Three small holes of 5 cm depth were made in the soil with a glass rod in a diameter of 0.5 cm from the anthurium stem, and 10 ml of nematode suspension was pippetted in total. The plants were managed as usual except the first 5 days without watering. Five replicates for each treatment were performed. After 56 days, nematodes were extracted from the roots and soil of all treated plants, counted and calculated as above, in addition, above-ground plant weight, root weight, and disease severity were recorded. Disease severity was classified into six levels from low to high according to the discolouration area on the roots: 0= no discolouration on the roots; 1=discolouration area less than 5 % on the roots; 2=discolouration area less than 25 % and more than 5 % on the roots; 3=discolouration area less than 50 % and more than 25 % on the roots; 4=discolouration area less than 75 % and more than 50 % on the roots; 5=discolouration area more than 75 % on the roots (Fig. 1).

Fig. 1 *Anthurium andraeanum* root infected with *Radopholus similis* compared with uninfected roots (0). 0=no discolouration of the roots; 1=<5 % discolouration; 2=5 %–25 % discolouration; 3=25–50 % discolouration; 4=50–75 % discolouration; 5=>75 % discolouration

Data analysis

All data in this study were subjected to analysis of variance (ANOVA) and multiple comparisons of means were conducted by Duncan's Multiple Range Test at $P=0.05$ using SAS (Release 8.01).

Results

Alignment and differences of the ITS sequences of two *R. similis* populations *Rs*-C and *Rs*-P

The length of the two ITS sequences (Accession number: JQ619539, JQ619538) of *Rs*-C and *Rs*-P were both 625 bp. There were two variant nucleotides in ITS sequence at 5.8 s different between *Rs*-C and *Rs*-P, at NO.373 and NO.402 respectively.

Isolation and identification of *Rs-eng-1b* of *R. similis*

Total RNA extracted from *Rs*-C and *Rs*-P nematode populations respectively were reverse transcribed into cDNA for SSH. A total of 2,400 clones were randomly selected from secondary round PCR products and validated to have an 89.2 % positive rate by PCR. Positive clones were made through reverse northern blotting and resulted in 124 hybridization spots with significantly different expression (Fig. 2). Finally, 124 positive clones were sequenced and 112 of them were successfully. After removing the vector sequences and the adaptor sequence, 87 unique sequences were obtained and aligned against a non-redundant nucleotide database and the non-redundant protein database in NCBI. The alignment results showed that five of the 87 sequences were homologous to *Rs-eng-1b*. Of the five sequences, a 467 bp (Accession number: GW395922) and a 742 bp (Accession number: GW395923) could be spliced into a sequence with a length of 1,183 bp.

According to the spliced 1,183 bp fragment of *Rs-eng-1b*, the nest primers were designed to perform 5′ RACE-PCR and 3′ RACE-PCR. Two fragments in length of 750 bp (5′ RACE) and 1,180 bp (3′ RACE) were obtained. Finally, the two fragments were spliced into a sequence with a length of 1,400 bp. The sequence compared with GenBank accessions showed 99 % similarity with *Rs-eng-1b* (Accession number: EU414839). Therefore, we conferred that the 1,400 bp sequence was *Rs-eng-1b* sequence of *R. similis.*

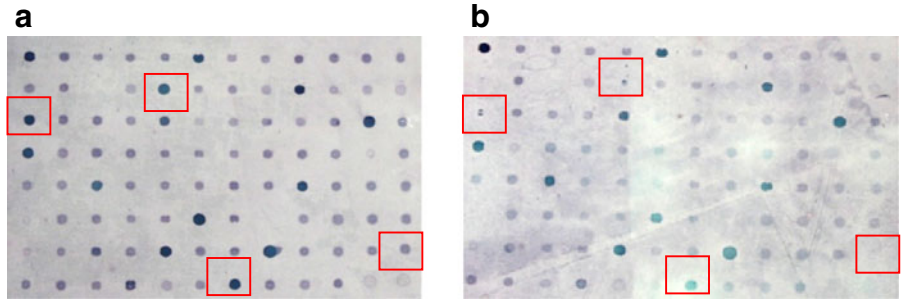

Fig. 2 cDNA Microarray analysis of differentially expressed genes between *Rs*-C and *Rs*-P populations of *Radopholus similis*. **a** Hybridization result with random cDNA probe of *Rs*-C; (**b**) Hybridization result with random cDNA probe of *Rs*-P. The *red blocks* show the positive clones of cDNA fragments differentially expressed between *Rs*-C and *Rs*-P population

Expression analysis of *Rs-eng-1b* in different populations and different development stages of *R. similis*

The qPCR results showed that the expression of *Rs-eng-1b* in *Rs*-C were about 2.7 times as much as the expression of *Rs*-P (Fig. 3). According to the expression of *Rs-eng-1b* in *Rs*-C eggs, females, juveniles, and males, *Rs-eng-1b* had highest expression levels in females (Fig. 4). The expression in juveniles, males and eggs accounted for 30.5 %, 15.5 %, and 10.2 % of the expression levels in females.

RNAi silence effect on *Rs-eng-1b* expression

After *Rs-eng-1b* dsRNA was synthesized, the RNAi silencing effect of *Rs-eng-1b* in *Rs*-C population was detected by qPCR methods. Compared with relative expression levels of *Rs-eng-1b* in corresponding control nematodes soaked in *gfp* dsRNA, the relative expression of *Rs-eng-1b* in nematodes soaked in *Rs-eng-1b* dsRNA decreased by 41.8 % for the 12 h treatment, 61.5 % for the 24 h treatment, 66.2 % for the 36 h treatment, and 62.3 % for the 48 h treatment (Fig. 5), difference were significant (P=0.05) between the nematodes treated with *Rs-eng-1b* dsRNA and the untreated nematodes, and nematodes treated with *gfp* dsRNA, respectively. In treatments by RNAi silencing of *Rs-eng-1b*, the silencing effect at 36 h and 48 h were significantly higher than the silencing effect at 12 and 24 h. Expression of *Rs-eng-1b* was inhibited by *Rs-eng-1b* dsRNA and the silencing effect was enhanced with increasing exposure time. The non-endogenous *gfp* dsRNA control had no significant influence on the expression of *Rs-eng-1b*.

Influence of *Rs-eng-1b*'s silence on reproduction and pathogenicity in *R. similis*

As the RNAi effect on *Rs-eng-1b* expression was clear, the effect of gene silencing on nematode

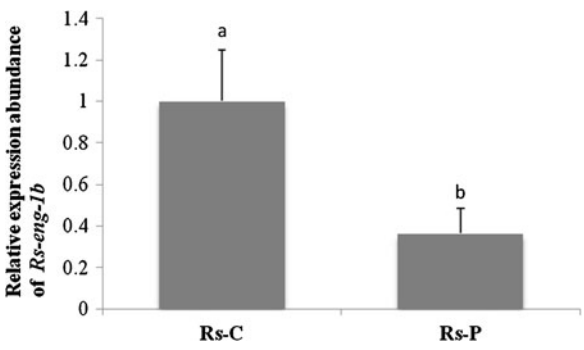

Fig. 3 Expression of the endoglucanase *Rs-eng-1b* in *Radopholus similis* populations *Rs*-C and *Rs*-P. *Bars* indicate standard errors of mean data (n=3) and *different letters* indicate significant differences (P=0.05) between treatments

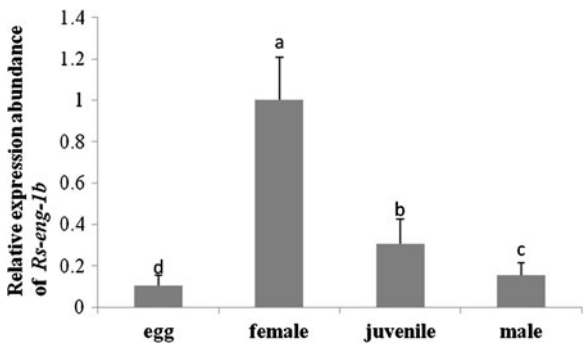

Fig. 4 Expression of the endoglucanase *Rs-eng-1b* in 100 eggs, females, juveniles, and males, respectively of *Radopholus similis* populations *Rs*-C. *Bars* indicate standard errors of mean data (n= 3) and *different letters* indicate significant differences (P=0.05) between treatments

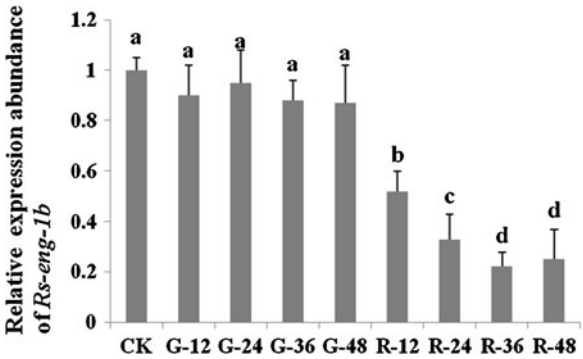

Fig. 5 Expression of the endoglucanase *Rs-eng-1b* in *Radopholus similis* population *Rs*-C treated with *Rs-eng-1b* double-stranded (ds) RNA. CK: expression of *Rs-eng-1b* in untreated nematodes; G-12, G-24, G-36, and G-48: expression of *Rs-eng-1b* in control nematodes soaked by non-endogenous *gfp* dsRNA solution for 12 h, 24 h, 36 h, and 48 h, respectively; R-12, R-24, R-36, and R-48: expression of *Rs-eng-1b* in nematodes soaked by *Rs-eng-1b* dsRNA for 12 h, 24 h, 36 h, and 48 h, respectively. *Bars* indicate standard errors of mean data (n=3) and *different letters* indicate significant differences (P=0.05) between treatments

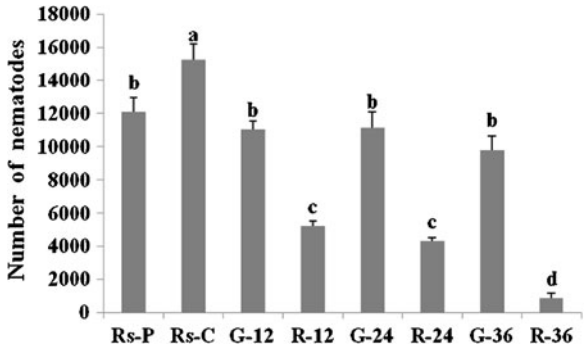

Fig. 6 Number of *Radopholus similis* on carrot callus 56 days after inoculation of 30 females, respectively. *Rs*-P and *Rs*-C: untreated population *Rs*-P and *Rs*-C; R-12, R-24, R-36: Number of *R. similis* after inoculating 30 females *Rs*-C treated by *Rs-eng-1b* dsRNA for 12 h, 24 h, and 36 h, respectively; G-12, G-24, and G-36: Number of *R. similis* after inoculating 30 females of *Rs*-C treated by non-endogenous *gfp* dsRNA solution for 12 h, 24 h and 36 h, respectively. *Rs*-C and *Rs*-P were two populations of *R. similis* collected from roots of ornamental plants *Calathea makoyana* and *Philodendron cv* Green Emerald, respectively. *Bars* indicate standard errors of mean data (n=5) and *different letters* indicate significant differences (P=0.05) among treatments

reproduction was studied by respectively inoculating treated and untreated nematodes on carrot callus. The results showed that after being soaked in *Rs-eng-1b* dsRNA and inoculated onto a carrot callus for 56 days (about two lifecycles of *R. similis*), *Rs*-C nematodes treated with *Rs-eng-1b* dsRNA for 12 h, 24 h, and 36 h had significantly lower (P=0.05) reproduction than untreated *Rs*-C and *Rs*-P nematodes, and *Rs*-C nematodes treated with *gfp* dsRNA, respectively (Fig. 6). The reproduction of nematodes treated with *gfp* dsRNA was significantly lower (P=0.05) than untreated nematodes and did not decrease with increasing length of exposure time. Nematodes without any treatment from *Rs*-C had the greatest reproduction. So, with exposure to *Rs-eng-1b* RNAi treatment increased, nematode reproduction decreased.

In addition, the RNAi effect of *Rs-eng-1b* on pathogenicity of *R. similis* was also studied by respectively inoculating treated and untreated nematodes to anthurium for 56 days. The results showed that above-ground plant weight and root weight of anthurium inoculated with either untreated *R. similis Rs*-C or *Rs*-P were significantly lower (P=0.05) than for non-nematode treated anthurium (Fig. 7a–b). Anthurium inoculated with untreated *Rs*-C had lower root weight and above-ground plant weight compared to plants inoculated with untreated *Rs*-P. The numbers of nematodes in the rhizosphere and the disease severity level of anthurium inoculated with untreated *Rs*-C were significantly higher (P=0.05) than for anthurium inoculated with untreated *Rs*-P (Fig. 7c–d).

Anthurium plants were inoculated with *Rs*-C nematodes that had been treated with either *Rs-eng-1b* dsRNA or *gfp* dsRNA, or with untreated *Rs*-C and *Rs*-P nematodes. After infection for 56 days, fresh anthurium above-ground plants and roots were weighed and compared. The results (Fig. 7a–b) showed that anthurium plants inoculated with the *Rs*-C nematodes treated with *Rs-eng-1b* dsRNA for 36 h had the heaviest above-ground plant and root weights. Its above-ground weight was significantly greater than those in other treatments (P=0.05), and its root weight was remarkably heavier (P=0.05) than those with *gfp* dsRNA treatments for 24 h and 36 h, as well as the untreated *Rs*-C and *Rs*-P nematodes. For above-ground plant weight of anthurium inoculated with *Rs*-C nematodes untreated and treated with *Rs-eng-1b* dsRNA for 12 h and 24 h, or with *gfp* dsRNA for 12 h, 24 h and 36 h, the difference in above-ground plant weight was only significant (P=0.05) between the untreated nematodes and the nematodes treated with *Rs-eng-1b* dsRNA for 24 h. Differences in root weight were significant (P=0.05) between the untreated nematodes and the nematodes treated with *Rs-eng-1b* dsRNA for 12 h and 24 h, and *gfp* dsRNA for 12 h,

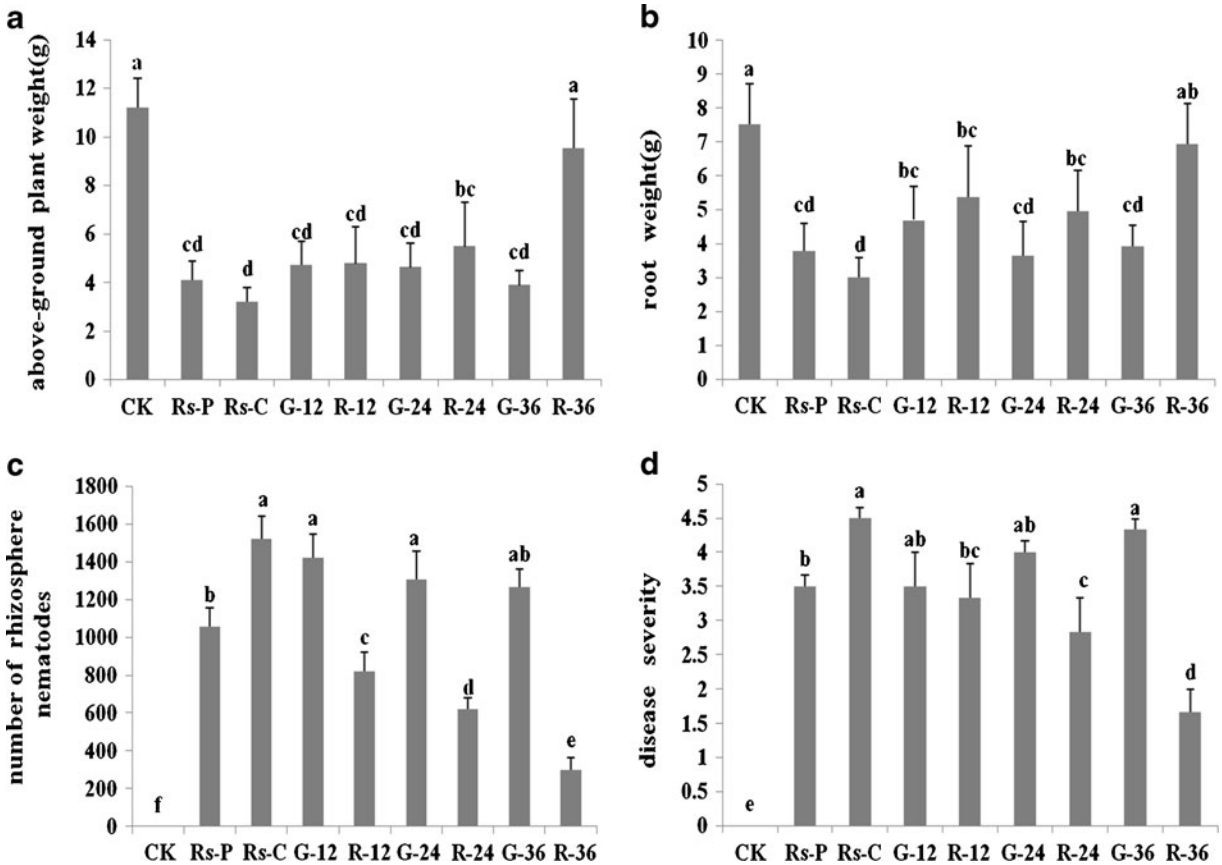

Fig. 7 Above-ground plant weight (**a**), root weight (**b**), number of nematodes in the rhizosphere (**c**), and disease severity (**d**) of *Anthurium andraeanum* at 56 days after being inoculated with *Radopholus similis*. CK: uninoculated control; *Rs*-P and *Rs*-C: untreated *Rs*-P and *Rs*-C populations; R-12, R-24, and R-36: inoculated *Rs*-C population treated with dsRNA of *R. similis Rs-eng-1b* for 12 h, 24 h, and 36 h, respectively; G-12, G-24, G-36: inoculated *Rs*-C population treated with non-endogenous *gfp* dsRNA solution for 12 h, 24 h and 36 h, respectively. *Bars*

respectively. Comparison of the nematode number in the rhizosphere and the disease severity of anthurium inoculated with nematodes in various treatments showed that anthurium inoculated with *Rs*-C nematodes treated with *Rs-eng-1b* dsRNA for 24 h and 36 h were significantly lower in the nematode number in the rhizosphere and the disease severity than those inoculated with other nematodes (P=0.05), and that of *Rs-eng-1b* dsRNA for 36 h was the lowest (P=0.05) (Fig. 7c–d). Nematode number in the rhizosphere and disease severity in anthurium inoculated with *Rs*-C nematodes treated with *Rs-eng-1b* dsRNA for 12 h was considerably lower than that in anthurium inoculated with untreated *Rs*-C nematodes (P=0.05). There was no significant difference in the nematode number in the rhizosphere and disease severity among the treatments of anthurium inoculated with *Rs*-C nematodes treated with *gfp* dsRNA for 12 h, 24 h and 36 h, respectively, and untreated *Rs*-C nematodes. Taken together, these results suggest that the pathogenicity of *R. similis* could be reduced markedly after treatment with target-specific *Rs-eng-1b* dsRNA for 12–36 h, and that of *Rs-eng-1b* dsRNA for 36 h was the lowest among them, whereas the pathogenicity of *R. similis* was not impacted upon treatment with non-specific target *gfp* dsRNA for 12–36 h. Nematode number in the rhizosphere and disease severity of anthurium inoculated with untreated *Rs*-C nematodes were significantly more than those of anthurium inoculated with untreated *Rs*-P (P=0.05). Therefore, the pathogenicity of *Rs*-C nematodes was significantly stronger than that of the *Rs*-P nematodes (P=0.05).

Discussion

During feeding and migration in the plant, parasitic nematodes inject enzymes through the stylet into plant tissue to degrade the cell wall. The most extensively studied nematode cell wall-degrading enzyme is β-1, 4-endoglucanase (EGase) (Haegeman et al. 2009). *Rs-eng-1b* is known as EGases which functions in degrading cellulose (Goellner et al. 2001), a key component in forming cell walls. In this study, *Rs-eng-1b* was isolated and obtained according to our constructed SSH library built from mixed-stage nematodes of *Rs*-C and *Rs*-P populations with different pathogenic ability on *Musa paradisiaca.* The sequence of *Rs-eng-1b* in here was 1,427 bp that had a 99 % similarity with the first report of *Rs-eng-1b* (EU414839) (Haegeman et al. 2008). The expression of *Rs-eng-1b* in *Rs*-C was about 2.7 times higher than the expression in *Rs*-P, and the pathogenicity of *Rs*-C was significantly greater than that of *Rs*-P. Consequently, the expression of *Rs-eng-1b* was positively correlated with the pathogenicity of *R. similis*. The expression of *Rs-eng-1b* in the nematodes treated by *Rs-eng-1b* dsRNA for more than 12 hr decreased significantly, and the *Rs-eng-1b* dsRNA treatment caused the nematodes to have a lower reproduction than *gfp* dsRNA treated and untreated *Rs*-C. Furthermore, the growth of anthurium inoculated by the nematodes treated by *Rs-eng-1b* dsRNA was better than that of untreated nematodes and nematodes treated with *gfp* dsRNA, and the effect of *Rs-eng-1b* RNAi treatment was enhanced with extending time of *Rs-eng-1b* RNAi treatment. In view of these results, it is assumed that a higher expression of *Rs-eng-1b* results in more degradation of cellulose, and therefore contributes to nematode invasion of the hosts resulting in more serious damage to the plant.

Analysis of the expression of *Rs-eng-1b* performed at different nematode stages of *Rs*-C showed that *Rs-eng-1b* expression was highest in females and lowest in eggs. Interestingly, the expression difference of *Rs-eng-1b* in females, males, juveniles, and eggs, conforms to their individual developmental features. The female and juvenile of *R. similis* are the infective forms which have a powerful stylet and well developed pharyngeal glands (Williams and Siddiqi 1973; Gowen et al. 2005). EGases is thought to be secreted in order to degrade cell walls of hosts during infection, and may help nematodes to migrate through the host tissue and absorb nutrition from there. So the female and juvenile of *R. similis* may secrete abundantly EGases for infection. In addition to infection, the female is also responsible for breeding, and therefore requires additional nutrients (Gowen et al. 2005). So, females may secrete more EGases than juveniles due to their higher activity in gaining nutrients. In contrast, males of *R. similis* have a degraded stylet and pharyngeal glands are non-parasitical (Williams and Siddiqi 1973; Luc 1987; Gowen et al. 2005). Eggs need the least nutrition due to their immobility. Smant et al. (1998) reported that the EGases gene was only expressd in the mobile stages of *G. rostochiensis*, namely in pre-parasitic and parasitic J2 and in adult males, but not in sedentary females. Our present findings and the results reported by Smant et al. (1998) indicate that EGases may be related to parasitism and nematode infection. Haegeman et al. (2008) reported the expression of EGases in different life stages of *R. similis* by a semi-quantitative RT-PCR. In their study *Rs-eng-1b* was expressed at a very low level in eggs, and higher levels in males and females, but not in juveniles. They concluded that other EGases not identified in juveniles might complement the role of *Rs-eng-1b*.

Employing RNAi to study gene function of nematodes, there are in principle three approaches to input dsRNA: microinjection, feeding approaches using bacteria with expression of dsRNA, and soaking nematodes in dsRNA (Urwin et al. 2002). However, the first two approaches are unrealistic: due to the small size of nematodes it is difficult to microinject dsRNA, nor do PPNs feed upon bacteria. Therefore RNAi input on PPNs is generally performed by soaking the nematodes for more than 4 h for effective gene silencing (Rosso et al. 2009). Chen et al. (2005) soaked *G. rostochiensis* in dsRNA of *Gr-eng-1* for 24 h, Bakhetia et al. (2007) soaked *H. glycines* in dsRNA of *Hg-eng-1* for 16 h and Cheng et al. (2010) soaked *Bursaphelenchus xylophilus* in dsRNA of *Bx-eng-1* for 24 h for the best RNAi effect. However as shown in this study, overlong periods can also cause decreased expression. In this study, *R. similis* soaked in dsRNA of *Rs-eng-1b* for 36 h, expression of *Rs-eng-1b* of *R. similis* decreased significantly. All these results indicate that RNAi treatment for *eng-1* in different nematodes requires 16 h to 36 h exposure to display an effective silencing. Haegeman et al. (2009) silenced the putative

endoxylanase in *R. similis* (*Rs-xyl1*) with dsRNA for 24 h and the expression of *Rs-xyl1* clearly reduced. Li et al. (2010) reported that the expression of the Cathepsin B in *R. similis* (*Rs-cb-1*) was clearly reduced after soaking *R. similis* in dsRNA of *Rs-cb-1* for 24 h, and the best silencing effect was obtained after RNAi treatment for 48 h. In this study, expression of *Rs-eng-1b* decreased significantly after soaking *R. similis* in dsRNA of *Rs-eng-1b* for 24 h, and treatments for 36 h had the best effect overall.

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