Expression of Arabidopsis pathogenesis-related genes during nematode infection

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SUMMARY

The expression pattern of pathogenesis-related genes *PR-1* to *PR-5* was examined in the roots and leaves of *Arabidopsis thaliana* plants on infection with beet-cyst (*Heterodera schachtii*) and root-knot (*Meloidogyne incognita*) nematodes. During *H. schachtii* parasitism of *Arabidopsis*, the expression of *PR-1*, *PR-2* and *PR-5*, which are considered to be markers for salicylic acid (SA)-dependent systemic acquired resistance (SAR), was induced in both roots and leaves of infected plants. In addition, the expression of *PR-3* and *PR-4*, which are used as markers for jasmonic acid (JA)-dependent SAR, was not altered in roots, but in the leaves of *H. schachtii*-infected plants, the expression *PR-3* was induced, whereas the expression of *PR-4* was down-regulated. During *M. incognita* infection of *Arabidopsis*, the expression of *PR-1*, *PR-2* and *PR-5* was highly induced in roots, as was *PR-3* to a lesser extent, but the expression of *PR-4* was not altered, indicating that infection with *M. incognita* activated both SA- and JA-dependent SAR in roots. However, all *PR* genes examined (*PR-1* to *PR-5*) were down-regulated in the leaves of *M. incognita*-infected plants, suggesting the suppression of both SA- and JA-dependent SAR. Furthermore, constitutive expression of a single *PR* in *Arabidopsis* altered the transcription patterns of other *PR* genes, and the over-expression of *PR-1* reduced successful infection by both *H. schachtii* and *M. incognita*, whereas the over-expression of *PR-3* reduced host susceptibility to *M. incognita* but had no effect on *H. schachtii* parasitism. The results suggest that fundamental differences in the mechanisms of infection by beet-cyst and root-knot nematodes differentially regulate *PR* protein production and mobilization within susceptible host plants.

INTRODUCTION

Plants possess both basal and inducible mechanisms to defend themselves against invading pathogens. Such mechanisms include the formation of cell wall polymers, secondary metabolites (phytoanticipins and phytoalexins) and proteins with antimicrobial activities (Slusarenko *et al.*, 2000; Van Loon *et al.*, 2006). Defence responses are triggered on recognition of elicitors derived from the invading pathogens. The local recognition events subsequently initiate signalling compounds, which spread systemically throughout the plant tissues from the infection site, finally establishing increased resistance to secondary infections in distal tissues (Durrant and Dong, 2004). This spread of resistance is collectively termed 'systemic acquired resistance' (SAR).

In several plant species, the establishment of SAR is associated with enhanced transcription of a large number of genes. A major class of these genes encodes the so-called pathogenesis-related (PR) proteins that were initially isolated in defence responses, but can also be up-regulated during compatible plant–pathogen interactions (Van Loon *et al.*, 2006). The PR proteins have been implicated in active defence and may play a role in restricting pathogen development and spread in the plant. They have been described in at least 17 families and are induced in response to oomycetes, fungi, bacteria, viruses, insects and nematodes (Van Loon *et al.*, 2006). Most PR proteins possess antimicrobial activities *in vitro* through hydrolytic activities on cell walls, contact toxicity and may be involved in defence signalling. However, other PR proteins are developmentally regulated and expressed in specific plant organs or tissues, and others are induced in response to environmental stimuli, such as wounding or cold stress. Plant resistance to pathogens involves the plant hormones jasmonic acid (JA) and salicylic acid (SA) (Dong, 1998; Thomma *et al.*, 2001). SA and JA appear to trigger defence responses against distinct sets of pathogens. SA-dependent signalling seems to be crucial for resistance against biotrophic pathogens (Delaney *et al.*, 1994; Reuber *et al.*, 1998) and, in *Arabidopsis thaliana*, activates defence genes such as *PR-1*, *PR-2* and *PR-5* (Thomma *et al.*, 1998). In contrast, JA-dependent resistance appears to be more effective

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against necrotrophic pathogens (Staswick *et al.*, 1998; Vijayan *et al.*, 1998), and involves increased expression of *PR-3*, *PR-4* and *PR-12* (defensin or *PDF1.2*) genes in Arabidopsis (Thomma *et al.*, 1998).

Cyst and root-knot nematodes are obligate sedentary endoparasites of plant roots. On hatching in soil, the motile, infective, second-stage juveniles (J2s) penetrate plant roots and migrate through the root until the appropriate root vascular cells to form feeding sites are recognized. Cyst and root-knot nematodes secrete effector molecules into these selected root cells to induce their de-differentiation into elaborate, multinucleate and metabolically active feeding cells, called syncytia and giant cells, respectively, that are essential to sustain nematode growth and development (Davis *et al.*, 2008). A relatively few (three to six) giant cells are formed around the root-knot nematode head through individual cell expansion with karyokinesis uncoupled from cytokinesis (Jones, 1981). In contrast, coordinated dissolution of walls adjacent to an initial syncytial cell gives rise to a syncytium induced by cyst nematodes that can incorporate many cells distal to the nematode.

Microarray analysis has revealed that the expression of multiple plant genes is altered in nematode infection sites, including the plant genes involved in cellular metabolism, cell wall synthesis, the cell cycle, signal transduction and defence responses, including *PR* genes (Alkharouf *et al.*, 2006; Gheysen and Mitchum, 2009; Ithal *et al.*, 2007a, b; Jammes *et al.*, 2005; Puthoff *et al.*, 2003; Szakasits *et al.*, 2009). It has been reported that the infection of Arabidopsis roots by the beet-cyst nematode, *Heterodera schachtii*, induces the expression of *PR-1*, *PR-2* and *PR-5* in host roots and of *PR-1* in shoots (Wubben *et al.*, 2008). However, the potential expression of *PR-2* and *PR-5* in Arabidopsis shoots during *H. schachtii* infection is still unresolved. The potential change in expression of the JA-dependent *PR* genes, *PR-3* and *PR-4*, in the roots and shoots of nematode-infected plants is also unknown. Moreover, the expression of Arabidopsis *PR* genes during infection with the root-knot nematode, *Meloidogyne incognita*, and the role of *PR* genes in host–nematode interaction remain to be investigated. In this study, a more comprehensive analysis of the expression patterns of *PR* genes in the roots and leaves of Arabidopsis plants during infection with *H. schachtii* and *M. incognita* is presented. In addition, the effect of *PR* proteins on nematode parasitism was examined using transgenic Arabidopsis plants that over-express *PR* genes.

RESULTS

Expression of Arabidopsis *PR* genes during beet-cyst nematode infection

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to measure the transcript levels of selected

representatives (Seo *et al.*, 2008) of five classes of Arabidopsis *PR* genes (*PR-1*, *PR-2*, *PR-3*, *PR-4* and *PR-5*) in the roots and leaves of wild-type Arabidopsis plants at 0, 5, 9 and 14 days post-infection (dpi) with *H. schachtii*. *PR-1*, *PR-2* and *PR-5* are commonly used as molecular markers for SA-dependent SAR signalling and their expression is coordinately regulated by SA (Bowling *et al.*, 1994; Cao *et al.*, 1994; Uknes *et al.*, 1993), whereas *PR-3* and *PR-4* are often used as markers for JA-dependent SAR signalling. Therefore, expression analysis of these genes during nematode parasitism will indicate how SA- and JA-dependent SAR are manipulated during nematode parasitism. Our results showed that the expression of *PR-1* and *PR-2* genes increased in roots of *H. schachtii*-infected plants at 9 dpi and then declined at 14 dpi (Fig. 1A), whereas the expression of *PR-5* increased at 9 dpi and remained elevated at 14 dpi. In contrast, the expression levels of *PR-3* and *PR-4* did not change in roots of *H. schachtii*-infected plants (Fig. 1A). These results indicate that parasitism by the beet-cyst nematode elicits the SA-dependent SAR pathway in Arabidopsis roots and does not alter the JA-dependent SAR pathway.

In leaves of *H. schachtii*-infected plants (Fig. 1B), the expression of *PR-1* and *PR-2* followed a pattern similar to that observed in roots; their expression increased at 9 dpi and then declined thereafter. In addition, the expression of *PR-5* also increased at 9 dpi before it declined. These results indicate that parasitism by *H. schachtii* induces the SA-dependent SAR pathway in leaves of infected plants. In addition, JA-dependent SAR also appeared to be induced in leaves of *H. schachtii*-infected Arabidopsis, as indicated by an increase in *PR-3* mRNA transcript level, although *PR-4* expression appeared to be down-regulated (Fig. 1B). Taken together, these results indicate that *H. schachtii* induces SA-dependent SAR in both roots and leaves of infected plants and may alter JA-dependent SAR in leaves.

Expression of Arabidopsis *PR* genes during root-knot nematode infection

In addition to examining the expression of Arabidopsis *PR* genes during beet-cyst nematode parasitism, the expression patterns of Arabidopsis *PR-1*, *PR-2*, *PR-3*, *PR-4* and *PR-5* genes were also examined at 0, 5, 9 and 14 dpi with the southern root-knot nematode *M. incognita*. qRT-PCR analysis indicated that the expression of *PR-1*, *PR-2*, *PR-3* and *PR-5* increased in roots of infected plants at 9 dpi and then declined at 14 dpi, indicating that root-knot nematode infection induces both SA- and JA-dependent SAR in roots of infected plants (Fig. 2A), although the expression level of *PR-4* did not change. Interestingly, and contrary to *PR* expression in root tissues, the transcript levels of all *PR* genes (*PR-1* to *PR-5*) seemed to be down-regulated in leaves of *M. incognita*-infected plants (Fig. 2B), suggesting that

Fig. 1 Time-course analysis of pathogenesis-related (PR) gene expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in roots (A) and shoots (B) of wild-type *Arabidopsis* plants at 0, 5, 9 and 14 days post-infection (dpi) with the beet-cyst nematode, *Heterodera schachtii*. The presented data are the mean fold changes $\pm$ standard errors in PR transcript levels relative to uninfected control tissue (baseline set at 1.0) from three biological replicates. An asterisk (*) indicates that the mean fold change is significantly different from 1.0 as determined by *t*-test ($P < 0.05$).

M. incognita may be suppressing SA- and JA-dependent SAR in leaves of infected plants.

Effect of PR over-expression on nematode infection

To examine the potential ability of PR proteins to defend host plants against nematode infection, transgenic *Arabidopsis* plants were used that constitutively expressed *PR-1*, *PR-2*, *PR-3*, *PR-4* or *PR-5* (PR-OE) under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Seo *et al.*, 2008). Homozygous lines were generated and challenged with infective J2s of *H. schachtii* or *M. incognita* in six-well plates containing agar-based plant growth

medium. There was no apparent difference in growth between plants that over-expressed PR genes and wild-type control plants (data not shown). The numbers of developing (fourth-stage) *H. schachtii* females or root galls induced by *M. incognita* in wild-type and transgenic plants were counted 3–4 weeks post-infection to assess the parasitic success of nematodes in PR-OE roots.

In our assay, *Arabidopsis* plants that over-expressed *PR-1* and *PR-3* were less susceptible to infection by *H. schachtii* relative to nontransformed wild-type plants or plants transformed with the 35S::GUS gene (Fig. 3A). However, there was no difference in the number of developed cyst females in *Arabidopsis* roots that

Fig. 2 Time-course analysis of pathogenesis-related (*PR*) gene expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in roots (A) and shoots (B) of wild-type *Arabidopsis* plants at 0, 5, 9 and 14 days post-infection (dpi) with the southern root-knot nematode, *Meloidogyne incognita*. The presented data are the mean fold changes $\pm$ standard errors in *PR* transcript levels relative to uninfected control tissue (baseline set at 1.0) from two biological replicates. An asterisk (*) indicates that the mean fold change is significantly different from 1.0 as determined by *t*-test ($P < 0.05$).

over-expressed *PR-2*, *PR-4* and *PR-5* and in roots of nontransformed control plants (Fig. 3A). Plants that over-expressed *PR-1* were more resistant to root-knot nematode infection relative to the wild-type, as evidenced by the smaller number of galls developed relative to those in nontransformed control plants (Fig. 3B). No difference was observed between the number of galls developed on plants over-expressing *PR-2*, *PR-3*, *PR-4*, *PR-5* and wild-type control plants. These results indicate that constitutive expression of *PR-1* reduces the level of parasitism of both *H. schachtii* and *M. incognita*, whereas *PR-3* over-expression reduces only *H. schachtii* infection and has no effect on *M. incognita* development.

Effect of over-expression of a single *PR* gene on the transcriptional regulation of other *PR* genes

To assess whether constitutive expression of a single *PR* gene affects the transcript levels of other *PR* genes, we used qRT-PCR to measure the mRNA transcript levels of *PR-1*, *PR-2*, *PR-3*, *PR-4* and *PR-5* in *PR*-OE plants. As a gene marker of SA-dependent SAR, over-expression of *PR-1* augmented the transcriptional level of *PR-2* and, to a lesser extent, of *PR-5*, but had no effect on the transcription of *PR-3* and *PR-4* (Fig. 4). However, over-expression of *PR-2* reduced the transcription of *PR-1* but had no effect on the transcription of *PR-3*, *PR-4* and *PR-5*. These results

Fig. 3 Cyst and root-knot nematode infection of roots of transgenic *Arabidopsis thaliana* plants that over-expressed (OE) individual pathogenesis-related (PR) genes. (A) Number of beet cyst nematodes, *Heterodera schachtii*, that developed to fourth-stage juvenile (J4) in roots of *Arabidopsis* that over-expressed *PR-1–PR-5* genes. (B) Number of galls induced by the southern root-knot nematode, *Meloidogyne incognita*, in roots of *Arabidopsis* that over-expressed *PR-1–PR-5* genes. Data are the mean $\pm$ standard error ($n = 20$) number of J4 or galls, respectively, for PR-OE relative to wild-type Columbia-0 (WT). An asterisk (*) indicates that the mean is significantly different ($P < 0.05$) from WT in a paired *t*-test.

indicate that the over-expression of a component of SA-dependent SAR can alter the transcriptional regulation of other genes in the same pathway, but appears to have no effect on JA-dependent SAR. However, with regard to the PR markers of JA-dependent SAR, both *PR-3* and *PR-4* over-expression augmented the transcriptional level of *PR-1* and *PR-2*, but had no effect on *PR-5* expression, indicating that the activation of the JA-dependent SAR pathway induces the activation of SA-dependent SAR. Interestingly, over-expression of *PR-3* had no effect on *PR-4* expression, and over-expression of *PR-4* had no effect on the transcript level of *PR-3*, indicating that, although *PR-3* and *PR-4* are used as markers for the activation of JA-dependent SAR, they do not appear to be coordinately

expressed. Moreover, the over-expression of *PR-3*, *PR-4* and *PR-5* elevated the mRNA transcript of *PR-1* to a level comparable with or higher than the level of *PR-1* transcripts in PR1-OE plants. Likewise, the transcript level of *PR-2* is higher in plants over-expressing *PR-1* and *PR-4* than in PR2-OE plants.

DISCUSSION

Cyst and root-knot nematodes are sedentary endoparasites that secrete effector molecules into selected host root cells and transform them into metabolically active feeding sites that are crucial for the survival of the nematode (Davis *et al.*, 2008). Both cyst and root-knot nematodes alter the expression of defence-related

Fig. 4 Effect of over-expression of a single pathogenesis-related (*PR*) gene in transgenic Arabidopsis on the expression of other *PR* genes in whole-plant samples. The transcript level was measured by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in seedlings of wild-type Arabidopsis plants. The presented data are the mean fold changes $\pm$ standard errors in *PR* transcript levels relative to uninfected control tissue (baseline set at 1.0) from three biological replicates. An asterisk (*) indicates that the mean fold change is significantly different from 1.0 as determined by *t*-test ($P < 0.05$).

genes, possibly to escape host defence mechanisms (Gheysen and Mitchum, 2009; Ithal *et al.*, 2007a, b; Jammes *et al.*, 2005; Puthoff *et al.*, 2003; Szakasits *et al.*, 2009). The aim of this study was to provide a systematic characterization of the expression patterns of five classes of Arabidopsis *PR* genes during nematode interactions, and to assess the effect of the constitutive expression of *PR* genes on nematode parasitism.

qRT-PCR analysis indicated that *H. schachtii* elicits SA-dependent SAR in both roots and leaves of Arabidopsis-infected plants, as evidenced by an increase in transcript levels of *PR-2* and *PR-5*, which are used as markers of SA-dependent SAR. The induction of *PR-2* and *PR-5* in roots of *H. schachtii*-infected plant has been reported previously (Wubben *et al.*, 2008). However, contrary to that report, our results indicate that *PR-1* is also induced in roots of *H. schachtii*-infected plants.

JA-dependent SAR also appears to be modulated in *H. schachtii*-infected plants, but only in leaves. The up-regulation of *PR-3* and the down-regulation of *PR-4* in leaves of *H. schachtii*-infected plants indicate that, during nematode parasitism, these two genes are not coordinately regulated. Moreover, the expression of *PR-3* and *PR-4* in leaves, but not in roots, is surprising, given that the roots are in direct contact with the nematodes; it suggests that, during *H. schachtii* infection, the regulation of these two genes may be under different control mechanisms in leaves vs. roots.

By contrast, *M. incognita* appears to induce both SA-dependent and JA-dependent SAR in roots of infected plants, although we did not observe any changes in the transcriptional level of *PR-4*. The high levels of mRNA transcripts of *PR-2*, *PR-3* and *PR-5* observed in roots of *M. incognita*-infected plants relative to *H. schachtii*-infected roots could be attributed to the difference in the level of infectivity and/or to the different

mechanisms of parasitism used by these two different nematodes (Davis *et al.*, 2008). Contrary to *H. schachtii*, *M. incognita* seems to down-regulate both SA- and JA-dependent SAR in leaves of infected plants, as evidenced by the reduced levels of *PR-1* to *PR-5* transcripts. The down-regulation of *PR* genes following infection with root-knot nematodes has been documented previously in tomato (Sanz-Alferez *et al.*, 2008). In contrast, in the *Hero*-mediated incompatible response to potato cyst nematode, the up-regulation of *PR-1* and *PR-5* in roots was observed (Sobczak *et al.*, 2005). Furthermore, the difference in *PR* expression between the roots and leaves of *M. incognita*-infected Arabidopsis plants further supports our hypothesis that, during nematode parasitism, the expression of some *PR* genes may be under different control mechanisms in leaves vs. roots. In a recent study, it has been reported that the regulation of *PR* genes is different in seedlings relative to adult plants (Fiocchetti *et al.*, 2006). It is worth noting that our analysis of *PR* expression reflects expression in whole root or shoot systems and does not indicate transcriptional changes at the nematode feeding sites. Therefore, we cannot rule out the possibility that the expression of *PR* genes analysed in this study may be under different regulatory mechanisms directly in nematode-feeding sites.

The similar spike in induction of *PR-1*, *PR-2* and *PR-5* at 9 dpi in the roots by both root-knot and cyst nematodes is curious, given the differential ontogeny of giant cells and syncytia, respectively (Jones, 1981). The early stages (0–6 days) of giant cell formation are characterized by cell elongation, expansion and nuclear division, compared with the coordinated cell wall dissolution characteristic of syncytia at similar time points. Giant cells are essentially mature by around 10–12 days after initiation, whereas syncytia continue to grow for up to 3–4 weeks, together with the developing cyst nematode. At the 9-day time

point, however, the osmoticum and cell wall thickening of both giant cells and syncytia increase to dramatic levels to support the feeding nematodes (Jones, 1981). Interestingly, the potential functions of *PR-2* and *PR-5* genes can include wall modifications and osmotic regulation (Van Loon *et al.*, 2006), respectively, which may account for their similar expression patterns in roots at 9 dpi by either root-knot or cyst nematodes.

The over-expression of PR proteins in transgenic plants has been used previously to increase tolerance to plant pathogens. For instance, increased tolerance to *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* was demonstrated in tobacco over-expressing the *PR-1a* gene (Alexander *et al.*, 1993). Transgenic rice and orange plants over-expressing *PR-5* possess increased tolerance to *Rhizoctonia solani* and *Phytophthora infestans* (Bachmann *et al.*, 1998). *PR-2* and *PR-3* genes confer resistance of carrot to several pathogens. The simultaneous expression of tobacco β -1,3-glucanase and chitinase genes in tomato plants results in increased resistance to fungal pathogens (Melchers *et al.*, 1998). Moreover, it has long been suggested that the differences between susceptibility and resistance are associated with differences in the timing and magnitude of defence changes (Tao *et al.*, 2003). Thus, we hypothesized that the effectiveness of PR proteins against nematode infection may lie both in their magnitude and rapidity of onset. To test this hypothesis, we used transgenic Arabidopsis plants that expressed *PR-1* to *PR-5* under the control of the CaMV 35S promoter, and examined their response to nematode infection. Our results indicated that transgenic Arabidopsis plants that constitutively express *PR-3* exhibit reduced susceptibility to *H. schachtii* infection relative to wild-type plants or plants over-expressing *PR-2*, *PR-4* and *PR-5*, whereas *PR-1*-over-expressing plants exhibit reduced susceptibility to both *H. schachtii* and *M. incognita* infection. The mechanism of the increased resistance to nematodes is still unclear. Although chitin is not a known structural component of the nematode stages observed here (chitin is present only in the nematode eggshell), *PR-3* may potentially modulate the chitinase effectors predicted to be secreted by parasitic cyst nematode life stages (Gao *et al.*, 2003). Alternatively, the over-expression of *PR-3* drastically increased the expression levels of *PR-1*, indicating that constitutive expression of a component of the plant response to pathogens can influence the expression of at least some other defence genes, and interfere with their transcriptional regulation, a phenomenon that has been reported previously in other studies (Fiocchi *et al.*, 2006; Linthorst *et al.*, 1989). The biological activity of *PR-1* has long remained unclear, although decreased host susceptibility to *H. schachtii* infection following treatment with SA has been associated with increased levels of *PR-1* in the roots and shoots (Wubben *et al.*, 2008), and application of SAR elicitors to tomato plants has been shown to activate *PR-1* and *PR-2*, and to reduce plant susceptibility to root-knot nematode infec-

tion (Sanz-Alferez *et al.*, 2008). *PR-1* is one of several genes that are coordinately regulated during the plant defence response (Maleck *et al.*, 2000), and may be viewed as a molecular marker for those genes that are effective against the nematode (Wubben *et al.*, 2008).

The over-expression of a PR gene of the SA-dependent SAR pathway affects the expression regulation of other PR genes of the same pathway, but does not affect the expression of PR gene markers of the JA-dependent SAR pathway. However, the over-expression of a PR gene marker in JA-dependent SAR induces the expression of PR gene markers of the SA-dependent SAR pathway, indicating cross-talk between the two pathways. This observation is consistent with the cross-talk between SA- and JA-dependent pathways that has been reported in a number of plant-pathogen interactions (Van Loon *et al.*, 2006). Signalling by SA appears to be a critical component to achieve nematode suppression in both resistance gene-mediated (Li *et al.*, 2006) and normally compatible (Wubben *et al.*, 2008) plant-nematode interactions. A systematic study of PR genes between compatible and incompatible plant-nematode pathosystems may similarly reveal that the quantitative and temporal expression of critical PR proteins (such as PR-1) may regulate the success of nematode parasitism. By the combination of analyses of reporter gene constructs with technologies to quantify PR gene expression within and around nematode feeding cells (Ithal *et al.*, 2007b; Szakasits *et al.*, 2009), the potential biological significance of localized PR gene expression on nematode parasitic success can be assessed.

EXPERIMENTAL PROCEDURES

PR genes and plant materials analysed

A list of the Arabidopsis PR genes analysed in this study and the primer sets used for PCR is presented in Table 1. Representatives of the *PR-1*–*PR-5* families were chosen for comparison with existing data on their expression in different plant tissues and stages of plant development (Seo *et al.*, 2008). *Arabidopsis thaliana* seeds that constitutively over-express individual *PR-1*–*PR-5* genes (PR-OE) (Seo *et al.*, 2008) were kindly provided by Chung-Mo Park at Seoul National University, Seoul, South Korea. Seeds were surface sterilized and plated in six-well culture plates (Falcon, Lincoln Park, NJ, USA) containing Knops medium (Sijmons *et al.*, 1991), which was solidified with 0.8% Daishin agar (Brunschwig Chemie, Amsterdam, the Netherlands). Plates were placed in a growth chamber at 25 °C under 16 h of light and 8 h of dark for 2 weeks before inoculation with nematodes.

Nematode culture and sterilization

Pure cultures of *H. schachtii* (beet-cyst nematode) and *M. incognita* (southern root-knot nematode) were grown on the roots of

Protein name	Accession number	Activity	Primer sequence (5'–3')	Amplicon (bp)
PR-1	AT2G14610	Unknown	F: TTCTTCCTCGAAAGCTCAA R: TTGCAACTGATTATGGTTCCAC	434
PR-2	AT3G57260	β -1,3-Glucanase	F: GCAATGCAGAACATCGAGAA R: TCATCCCTGAACCTTCCTTG	124
PR-3	AT3G12500	Endo-chitinase	F: CGGGCATCATTCAAGTTCT R: GCAACAAGGTCAGGGTTGTT	432
PR-4	AT3G04717	Endo-chitinase	F: TGTTCTCCGACCAACACTG R: CAATGAGATGGCCTTGTTGA	404
PR-5	AT1G75040	Thaumatococcal-like	F: CGTACAGGCTGCAACTTGA R: AGTGAAGGTGCTCGTTTCGT	465
Actin	ATU42007	N/A	F: GCCCGGAGCAGCATGAAGAT R: GCTGGAAAGTGCAGGAAGC	100

Table 1 The *Arabidopsis thaliana* pathogenesis-related (PR) genes (based on Seo *et al.*, 2008) examined in this study and the specific primers used in quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

plants in pots in a glasshouse. Eggs of *H. schachtii* were collected from crushed cysts developed on roots of cabbage plants (*Brassica oleracea* var. *capitata*), as described previously (Goellner *et al.*, 2001). Eggs of *M. incognita* were extracted from roots of tomato plants (*Solanum lycopersicon* cv. Rutgers), as described previously (Hussey and Barker, 1973). Eggs were hatched over water at 28 °C for 48 h, after which the hatched parasitic J2s (pre-J2s) were collected and surface sterilized by incubation for 10 min in sterilization solution (0.004% mercuric chloride, 0.004% sodium azide and 0.002% Triton X-100), followed by three washes with sterile distilled water.

Nematode infection assay and data collection

Seeds of wild-type *Arabidopsis* (ecotype Columbia) and PR-OE plants were surface sterilized and transferred (one seed per well) into six-well culture plates (Falcon) containing 6 mL of sterile modified Knops medium (Sijmons *et al.*, 1991) solidified with 0.8% Daishin agar (Brunschwig Chemie). Plates were placed in a 24 °C growth chamber under a 16 h light/8 h dark cycle for 2 weeks. After sterilization, J2 nematodes were suspended in 1.5% low-melting-point agarose to allow even distribution and to facilitate their movement into solid Knops medium. Plants were inoculated with approximately 60 J2s per plant and placed back in the growth chamber. The root systems of wild-type *Arabidopsis* were harvested at 0, 5, 9 and 14 dpi with either *H. schachtii* or *M. incognita*, and stored at –80 °C for subsequent RNA extraction and qRT-PCR. To analyse nematode infection rates of PR-OE plants, the cysts (for beet-cyst nematodes) and galls (for root-knot nematodes) developed in wild-type and PR-OE plants were counted 3–4 weeks post-infection, respectively, using a dissecting microscope, and the mean and standard error of 20 replicates per treatment were calculated. Statistical differences in the mean ($n = 20$) were determined by the paired *t*-test with an α level of 0.05 using SAS software (Cary, NC, USA).

RNA isolation and qRT-PCR

Total RNA for qRT-PCR was isolated from the bulked root systems of 12 *Arabidopsis* plants to achieve one biological rep-

licate, with two and three biological replicates completed for root-knot and cyst nematode-infected plants, respectively. Total RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. Prior to qRT-PCR, RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA) to eliminate any contaminating genomic DNA. First-strand cDNA was synthesized from 2–3 µg of total RNA using Super-Script II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-dT₁₈ primers following the manufacturer's instructions.

A single 20-µL PCR included 1 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2 µL of cDNA template and 5 µM each of forward and reverse primers. The PCR cycling parameters were set at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After completion of the cycling parameters, dissociation melt curve analysis (60–90 °C every 0.5 °C for 1 s) was conducted to discount the effects of primer dimer formation and contamination. The qRT-PCRs were performed in triplicate and the negative controls included water and mRNA prior to reverse transcription. The *Arabidopsis* actin 8 gene (ATU42007) was used as an internal control for the levels of cDNA used.

The relative fold change was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The paired *t*-test with an α level of 0.05 was used to compare the relative transcript level means employing the statistical software package of SAS. All qRT-PCRs were performed in a DNA Engine Opticon2 (Biorad, Hercules, CA, USA). Reactions were repeated at least twice and a representative result was displayed for individual assays. In addition to analysing PR gene expression in nematode-infected *Arabidopsis* plants, PR gene expression from RNA extracts of whole 2-week-old *Arabidopsis* plants of the different PR-OE *Arabidopsis* lines examined was also analysed by qRT-PCR using the conditions above.

ACKNOWLEDGEMENTS

This research was supported by the North Carolina Agricultural Research Service and by funding through the US Department of

Agriculture-National Institute of Food and Agriculture (USDA-NIFA) grant 2008-35302-18824.

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