MOLECULAR PLANT PATHOLOGY (2004) **5**(0), 000–000 DOI: 10.1111/J.1364-3703.2004.00216.X

The promoter of the *Arabidopsis thaliana Cel1* endo-1,4-β glucanase gene is differentially expressed in plant feeding cells induced by root-knot and cyst nematodes

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SUMMARY

Transgenic tobacco and *Arabidopsis thaliana* carrying the *Arabidopsis* endo-1,4-β-glucanase (EC 3.2.1.4) *Cel1* promoter fused to the β-glucuronidase (GUS) reporter gene were infected with the root-knot nematode, *Meloidogyne incognita*, and either the tobacco cyst nematode, *Globodera tabacum* (tobacco), or beet cyst nematode, *Heterodera schachtii* (*Arabidopsis*). *Cel1*-driven GUS expression was detected in cell elongation zones of non-infected plants and within feeding sites (giant-cells) induced in roots of both plant hosts by *M. incognita*. The first detectable signs of *Cel1* expression within developing giant-cells occurred at the onset of giant-cell formation and continued throughout the *M. incognita* life cycle. *UidA* (*Gus*) transcripts were detectable within giant-cells induced in tobacco roots at 11–13 days post-inoculation with *M. incognita* as determined by *in situ* mRNA hybridization. By contrast, expression of the *Cel1* promoter was not detected within developing syncytia induced in tobacco or *Arabidopsis* roots by *G. tabacum* and *H. schachtii*, respectively, at any time point. The results demonstrate specific regulation of cell wall-degrading enzymes that may be required for cell wall modifications during feeding cell formation by sedentary endoparasitic nematodes. Differential expression of *Cel1* by cyst and root-knot nematodes further supports underlying mechanistic differences in giant-cell and syncytium formation.

INTRODUCTION

Root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes are obligate, sedentary endoparasites that alter the expression of numerous plant genes during feeding cell formation in susceptible host plant roots (Bird, 1996; Gheysen and Fenoll, 2002). The initiation of multinucleate feeding cells, called giant-cells, by *Meloidogyne* species occurs by karyokinesis without cytokinesis in 5–7 hypertrophied cells around the head of the nematode (Sijmons *et al.*, 1994). Individual giant-cells continue to enlarge both laterally and at their extremities for 2–3 weeks after induction (Jones, 1981). By contrast, cyst nematode species induce multinucleate feeding cells, called syncytia, by initially modifying a single cell and subsequently incorporating adjacent cells into the syncytium via extensive cell wall dissolution and protoplast fusion (Grundler *et al.*, 1998; Sijmons *et al.*, 1994). Although the mechanisms of giant-cell and syncytium formation differ, these nematode feeding cells have several characteristics in common, including: localization in vascular tissue adjacent to xylem elements, dense cytoplasm, endoreduplication, organelle proliferation, and elaborate thickening and ingrowths of peripheral cell walls (Hussey and Grundler, 1998).

The structure of giant-cells and syncytia suggests that extensive cell wall modifications are required for their formation and that plant hydrolases may play a role in the cell wall architectural modifications that occur during nematode-induced feeding cell formation (Hussey and Grundler, 1998; Jones and Dropkin, 1975). The plant cell wall is an extensive network of cellulose microfibrils, hemicelluloses, pectins and proteins that undergoes controlled (regulated) changes in architecture during plant growth and development (Carpita and Gibeaut, 1993). Plant endo-β-1,4-glucanases (EGases) are cellulolytic enzymes (cellulases) that catalyse the breakdown of β-1,4 glucosidic linkages and have been shown to be expressed during fruit ripening, senescence, and in elongating and expanding cells, suggesting a role in plant development (del Campillo and Bennett, 1996; del Campillo, 1999; Lashbrook *et al.*, 1994; Nicol *et al.*, 1998; Shani *et al.*, 1997). The *Arabidopsis thaliana Cel1* (*Atcel1*) gene encodes a 54-kDa

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elongation-specific EGase that belongs to family 9 glycosyl hydrolases (Shani *et al.*, 1997). Northern blot analysis detected *Cel1* transcripts in the elongating zones of flowering stems, but not in fully expanded leaves or in the basal internode of flowering stems of *A. thaliana* plants (Shani *et al.*, 1997). Transgenic tobacco (*Nicotiana tabacum*) and *Arabidopsis* carrying a *Cel1 promoter–GUS* construct demonstrated GUS expression specifically within both shoot and root elongation zones (Shani *et al.*, 1997; Tsabary, 2001), and in aspen (*Populus tremula*), the *A. thaliana Cel1* promoter was also expressed in elongating tissues (Shani *et al.*, 2000). Recent evidence suggests that both cyst and root-knot nematodes recruit plant EGase activity as one component of the extensive remodelling of cell walls that occurs within their respective feeding cells (Goellner *et al.*, 2001). It is hypothesized that a battery of cell wall-modifying enzymes gives rise to the differences and similarities in cell wall phenotype observed within syncytia and giant-cells (Goellner *et al.*, 2001; Hussey and Grundler, 1998; Jones and Dropkin, 1975; Vercauteren *et al.*, 2002), but it is unclear how these processes are temporally and spatially regulated. The *Atcel1* promoter is expressed in similar tissues among diverse plant species (Shani *et al.*, 1997, 2000; Tsabary, 2001), suggesting that it responds to common regulatory machinery within heterologous plant hosts. CEL1 from *Arabidopsis* plays an important role in cell wall relaxation during cell growth, expansion and normal cell wall development (Shani *et al.*, 1997; Tsabary, 2001; Tsabary *et al.*, 2003) all of which are attributes amenable to modification during the formation of feeding cells by both cyst and root-knot nematodes. *Atcel1* is also expressed specifically within the root elongation zone—the preferred initial infection site of both root-knot and cyst nematodes (Hussey and Grundler, 1998). We report here the effects of root-knot and cyst nematode infection on the expression of the *A. thaliana* elongation-specific *Cel1* promoter in transgenic tobacco and *Arabidopsis* roots.

RESULTS

In noninfected transgenic tobacco roots containing the *Atcel1* promoter–*GUS* construct (*Cel1-GUS*), GUS activity was only detected in the elongation zones of root tips (Fig. 1A) and emerging root initials. In nematode-infected *Cel1-GUS* tobacco roots, strong GUS expression was detected in the central tissues of developing galls induced by *Meloidogyne incognita* (Fig. 1B) and in noninfected root tips. *Cel1-GUS* activity and nematode development were monitored from 2 to 20 days after root penetration by *M. incognita* and *Globodera tabacum* second-stage juveniles (J2). Sectioning revealed faint, but specific *Cel1*-driven GUS expression within *M. incognita*-induced giant-cells as early as 4 days postinoculation with J2 (Fig. 1C). *Cel1-GUS* expression was detected specifically within giant-cells during the parasitic J2 through the adult female root-knot nematode life stages (Fig. 1D–G). Expression of *Cel1-GUS* was not detected histochemically at any parasitic stage within syncytia induced in transgenic tobacco by *G. tabacum* (data not shown). mRNA *in situ* hybridizations were conducted using a *UidA* riboprobe to determine whether the GUS expression observed within giant-cells during later stages of nematode development was due to newly synthesized GUS enzyme or stable enzymatic product produced during earlier time points. *UidA* transcripts were still detectable at 11–13 days postinoculation within developing giant-cells with an anti-sense *UidA* riboprobe (Fig. 1I), but not with the sense *UidA* riboprobe (Fig. 1H). *UidA* transcripts were also detected with the anti-sense *UidA* riboprobe in lateral root initials stimulated at the RKN infection site (data not shown), consistent with the observed *Cel1-GUS* expression in elongation zones of roots (Shani *et al.*, 1997).

GUS activity in expanding shoot tissues was used to verify *Cel1*-driven GUS expression in transgenic *Arabidopsis* seedlings that were not infected with nematodes (Fig. 2A). After infection of *Cel1-GUS Arabidopsis* roots with *M. incognita*, GUS activity was observed predominantly in the developing giant-cells (Fig. 2B) induced in roots by the nematodes. The expression patterns in *Arabidopsis* were similar in five independent *Cel1-GUS* transgenic lines. In transgenic tobacco containing the Δ0.6 Tob*RB7* promoter–GUS construct (Opperman *et al.*, 1994) treated similarly as a positive control, GUS activity was observed in the root elongation zone and within *Meloidogyne*-induced giant-cells. *Cel1-GUS* activity within giant-cells in *Arabidopsis* was observed as early as 3 days postinfection with *M. incognita* J2 and persisted throughout the root-knot nematode life cycle. Cross-sections of galls in *Cel1-GUS Arabidopsis* roots at 7 days postinfection confirmed GUS activity within the giant-cells induced by *M. incognita* (Fig. 2C). GUS staining was also localized to pericycle cells immediately surrounding giant-cells induced within galls of *Cel1-GUS Arabidopsis*, which was not observed in *Cel1-GUS* tobacco, and occasionally extended into adjacent vascular tissues (Fig. 2C). As observed with *G. tabacum*-infected *Cel1-GUS* tobacco, no GUS activity was observed within nor surrounding syncytia induced in *Cel1-GUS Arabidopsis* at any time point after inoculation with the sugarbeet cyst nematode, *Heterodera schachtii* (Fig. 2D).

Expression of the promoter of the *Arabidopsis thaliana Cel1* endoglucanase gene (Shani *et al.*, 1997; Tsabary, 2001) was up-regulated within giant-cells formed by root-knot nematodes infecting roots of transgenic tobacco and *Arabidopsis* that contained a *Cel1-GUS* construct. The up-regulation of the *Atcel1* promoter was detected within the early stages of giant-cell formation and persisted through the formation of mature giant-cells in both plant hosts. By contrast, no stimulation of *Atcel1* promoter activity was observed within syncytia induced by cyst nematodes in either host plant. The observed *Cel1-GUS* activity in root and shoot elongation zones of noninfected transgenic

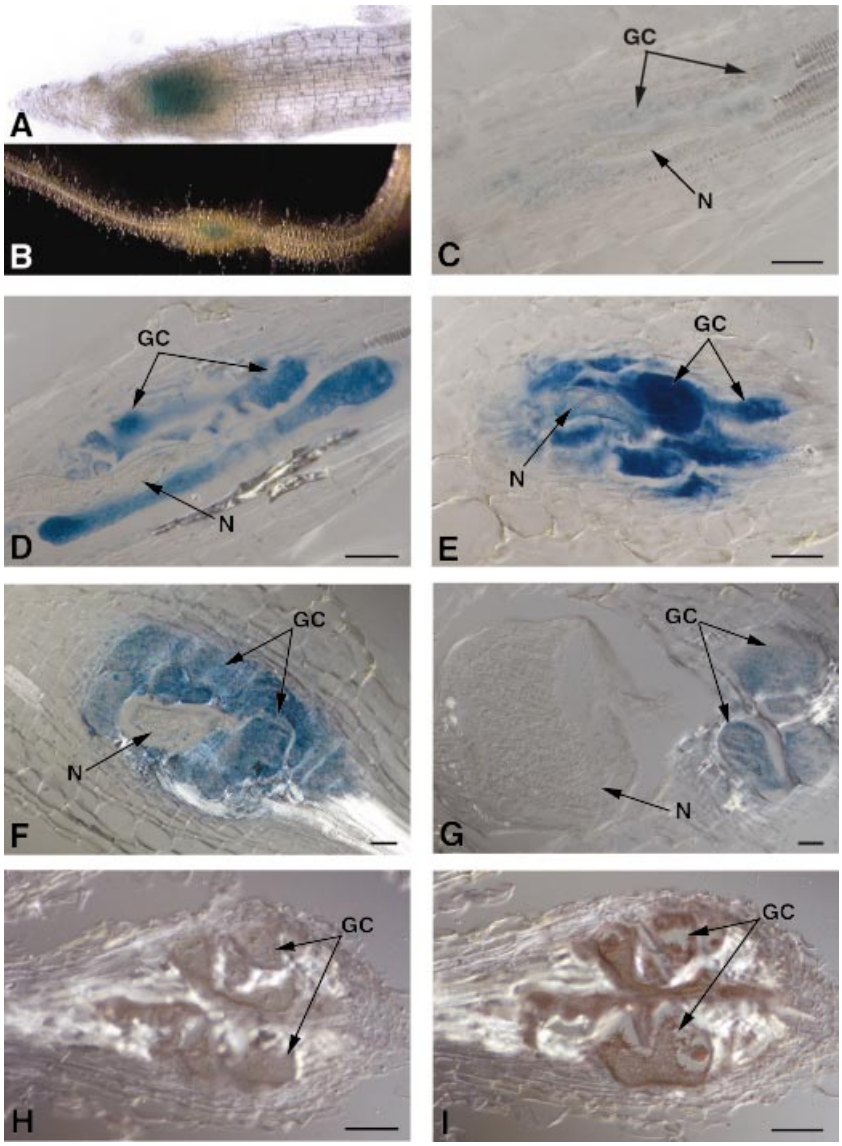

Fig. 1 Root knot nematode-induced expression of the *Arabidopsis thaliana* (*Cel1*) endoglucanase promoter–GUS fusion in giant-cells of transgenic tobacco. (A) *Cel1*-driven *GUS* expression in a noninfected tobacco root. GUS activity is limited to the zone of elongation in the root tip. (B) Whole-mount histochemical GUS assay of a *Meloidogyne incognita*-infected *Cel1-GUS* transgenic tobacco root. GUS activity is confined to the central region of the developing gall tissue. (C–G) Sections (30 μm thick) through *M. incognita*-infected *Cel1-GUS* tobacco roots after GUS staining. GUS expression is restricted to the giant-cells. (C) Second-stage juvenile (J2) initiating giant-cells during the early stages of infection. (D) Second-stage juvenile feeding from several giant-cells around its head. (E) Third-stage juvenile (J3). (F) Fourth-stage juvenile (J4). (G) Adult female nematode that has deposited eggs. Sections through *M. incognita*-infected roots at 11–13 days postinfection hybridized with a sense *UidA* (GUS) riboprobe (H) and an antisense *UidA* riboprobe (I). Purple staining corresponding to *UidA* transcripts is localized within developing giant-cells. N = nematode, GC = giant-cells. Scale bars = 50 μm.

tobacco and *Arabidopsis* (respectively), detection of Δ0.6 Tob*RB7* promoter–GUS activity (Opperman *et al.*, 1994) in giant-cells of tobacco-positive controls, and strong expression of *Cel1-GUS* in giant-cells of both tobacco and *Arabidopsis* indicated that the lack of detectable *Cel1-GUS* activity within syncytia was not due to technical difficulties.

DISCUSSION

Differential regulation of transcription based upon specific *cis*-acting elements within the *Atcel1* promoter sequence may explain the observed difference in response to root-knot and cyst nematodes. Nematode-responsive promoters have been identified from bacterial, viral and plant origin (Ehsanpour and Jones, 1996; Gheysen & Fenoll, 2002; Goddijn *et al.*, 1993; Hansen *et al.*, 1996; Niebel *et al.*, 1993; Opperman *et al.*, 1994; Urwin *et al.*, 1997), several of which have been shown to be differentially regulated by root-knot nematodes and cyst nematodes. For example, the tobacco extensin gene promoter and the *TobRB7* root-specific promoter are both up-regulated by root-knot nematodes, but not by cyst nematodes (Niebel *et al.*, 1993; Opperman *et al.*, 1994). The presence of elements within promoters like *TobRB7* (Opperman *et al.*, 1994) that can uncouple expression in root elongation zones from nematode-response is further evidence of the specificity of transcriptional regulation during feeding cell formation. Local concentrations of phytohormones can affect cell wall metabolism and elongation that may be related to specificity in regulatory pathways (Alexander and Grierson, 2002;

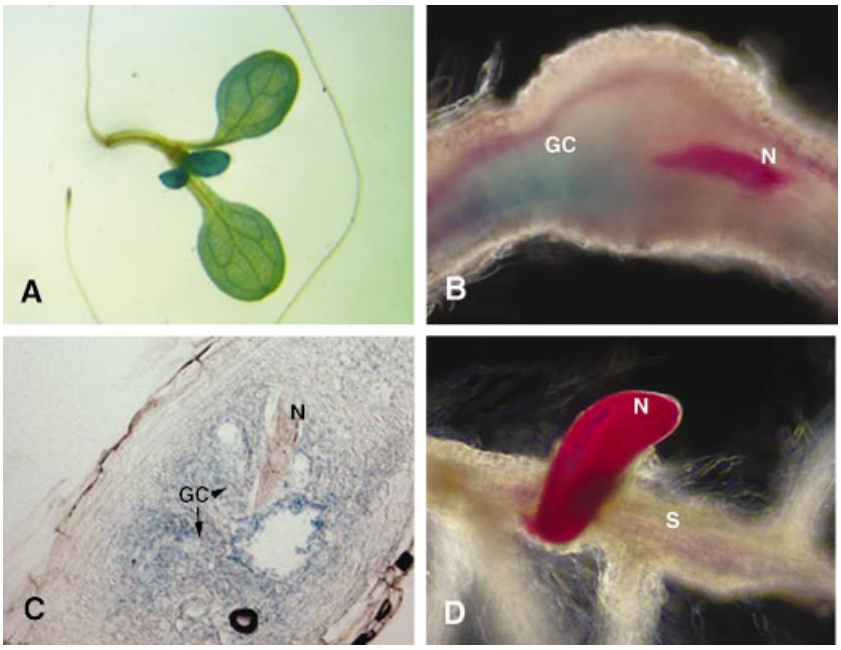

Fig. 2 Root knot nematode-induced *Cel1-GUS* expression in giant-cells of transgenic *Arabidopsis thaliana*. (A) *Cel1*-driven *GUS* expression in expanding shoot tissues of noninfected *A. thaliana* demonstrating the activity of the transgene. (B) Whole-mount histochemical GUS assay of a *Meloidogyne incognita*-infected *Cel1-GUS* transgenic *A. thaliana* root. GUS activity is confined to the giant-cell (GC) region of the developing gall tissue adjacent to the developing nematode (N) stained red with acid fuchsin. (C) Cross-section (15 μm thick) of an *M. incognita*-infected *A. thaliana* root demonstrating *Cel1*-driven GUS activity within giant-cells and the adjacent pericycle and vascular cells surrounding the nematode feeding site. (D) No detectable *Cel1-GUS* activity within an *Arabidopsis* root at 10 days after syncytium (S) development by a sugarbeet cyst nematode (N), *Heterodera schachtii* (stained with acid fuchsin).

Ecker, 1995; Vissenberg *et al.*, 2000). Phytohormone-mediated responses play a role in nematode feeding cell development (Gheysen and Fenoll, 2002; Goverse *et al.*, 2000), probably having both common and specific effects on *cis*-acting elements of genes encoding cell wall-modifying enzymes that give rise to giant-cells and syncytia.

That the *Atcel1* promoter is up-regulated in giant-cells of plant hosts from diverse families suggests that *cis*-acting elements in *Atcel1* respond to common regulatory factors in giant-cell development. The mechanism(s) by which nematodes influence plant cell differentiation is expected to have common regulatory features in different plant species, because a range of sedentary nematode species are able to develop feeding cells in numerous host plants (Davis *et al.*, 2000; Gheysen and Fenoll, 2002; Goddijn *et al.*, 1993). The evidence that the *Atcel1* promoter is up-regulated in giant-cells of *Arabidopsis* suggests the expression of a specific endoglucanase in heterologous hosts. This observation is supported by the recent identification of a small group of tobacco EGase genes including *Ntcel2*, *Ntcel4*, *Ntcel5*, *Ntcel7* and *Ntcel8* that are up-regulated during giant-cell development (Goellner *et al.*, 2001). Interestingly, the same suite of plant EGase genes are also up-regulated within syncytia in tobacco by cyst nematodes (Goellner *et al.*, 2001). Of the tobacco EGases up-regulated by cyst and root-knot nematodes, the *A. thaliana Cel1* has the highest amino acid identity (78%) with the tobacco *Cel2* endo-1,4-β-glucanase (Goellner *et al.*, 2001), but the biological significance of this similarity is unclear at present. This observation further suggests the presence of nematode-responsive *cis*-acting elements within the *Atcel1* promoter and that differential expression of plant EGase genes may be one measure of control of feeding cell phenotype. Giant-cells and syncytia are formed by fundamentally different mechanisms—it is possible that CEL1 plays an essential role in giant-cell development but not in syncytium formation, including potentially different requirements for feeding cell expansion.

The complex temporal expression and localization of cell wall-modifying enzymes that promote wall expansion in giant-cells and wall dissolution within syncytia, yet promote cell wall thickening and ingrowths in both types of feeding cells, probably represent modifications of mechanisms active during normal plant cell wall growth and maturation. EGases act in concert with a suite of plant cell wall-modifying enzymes, including xyloglucan endotransglycosylases and expansins, to maintain cell wall extensibility for cell expansion and elongation during various stages of plant growth and development (Cosgrove, 1999). Recently, an *Arabidopsis* pectin acetylesterase (PAE) gene was shown to be up-regulated in giant-cells and more weakly in syncytia (Vercauteren *et al.*, 2002). PAEs catalyse the deacetylation of pectin, making it more vulnerable to pectin-degrading enzymes such as pectate lyases, and they have been proposed to play a role in cell wall softening in nematode-infected roots (Vercauteren *et al.*, 2002). Altered expression of a number of plant genes related to cell wall modifications have been detected in nematode feeding cells (Gheysen and Fenoll, 2002). To assess the potential function of CEL1 in giant-cells, *A. thaliana* anti-sense *Cel1* plants expressing reduced levels of the CEL1 protein have been generated (Tsabary *et al.*, 2003) and studies are currently under way to examine the development of nematode feeding cells in these plants.

EXPERIMENTAL PROCEDURES

Transgenic tobacco (*Nicotiana tabacum* SR1) and *Arabidopsis thaliana* (Col-0 Ecotype) plants containing the *Arabidopsis Cel1* promoter fused to the β-glucuronidase (GUS) reporter gene were developed previously (Shani *et al.*, 1997; Tsabary, 2001). The construct was a transcriptional fusion between 1.6 kb of the putative *Cel1* promoter region (bases 5–1618; GenBank accession no. X98543) and the 5′ end of the β-glucuronidase gene (*UidA*; Jefferson, 1987; Shani *et al.*, 1997). Seeds of transgenic tobacco containing the Δ0.6 Tob*RB7* promoter–GUS construct (Opperman *et al.*, 1994) were a gift from C. H. Opperman (North Carolina State University). Seeds of all transgenic tobacco and *Arabidopsis*, and nontransformed (negative control) seeds of each species, were surface-sterilized with 2.5% sodium hypochlorite and 0.5% sodium dodecyl sulphate (SDS) for 10 min, rinsed four times with sterile water, and then germinated and grown monoxenically in Petri plates containing 0.8% Noble agar (Fisher Scientific, Pittsburgh, PA) supplemented with MS basal medium (Murashige and Skoog, 1962), pH 5.8, sucrose (30 g/L) and kanamycin (50 μg/mL). Tobacco and *Arabidopsis* seedlings were grown in a controlled temperature growth chamber at 25 °C with a 16-h photoperiod. At least five independent kanamycin-resistant lines were analysed for *Cel1-GUS* expression.

The root-knot nematode, *Meloidogyne incognita* Race 4, was propagated on greenhouse-grown tomato (*Lycopersicon esculentum* cv. Rutgers). *M. incognita* eggs were isolated from egg masses on tomato roots with 0.5% sodium hypochlorite (Hussey and Barker, 1973), stirred in a solution of 0.02% sodium azide for 30 min, rinsed with water on a 25-μm sieve, and then hatched over water containing 1.5 mg/mL gentamycin sulphate and 0.05 mg/mL nystatin at 28 °C on a Baermann pan. The tobacco cyst nematode, *Globodera tabacum* subspecies *solanacearum*, and the beet cyst nematode, *Heterodera schachtii*, were propagated on greenhouse-grown tobacco (*N. tabacum* cv. NC95) and cabbage (*Brassica oleracea* cv. All Season), respectively. To isolate *G. tabacum* and *H. schachtii* eggs, the cysts were gently crushed in a glass homogenizer and the eggs were collected and rinsed in water on to a 25-μm sieve. Nematode eggs were hatched on a Baermann pan at 27 °C in water or in filter-sterilized tobacco root diffusate (*G. tabacum* only) as previously described (Goellner *et al.*, 2000; LaMondia, 1995). After 2 days, hatched second-stage juveniles (J2) of *M. incognita, G. tabacum* and *H. schachtii* were collected and rinsed with water on a 25-μm sieve, surface sterilized with 0.002% mercuric chloride, 0.002% sodium azide and 0.001% Triton X-100 for 10 min, and then rinsed five times with sterile water. Surface-sterilized *M. incognita* J2 were resuspended in 50 μL of 2 mM Penicillin-G and 950 μL of 0.1% water agar at a concentration of 100 J2/10 μL for *Arabidopsis* inoculations and 10 J2/10 μL for tobacco inoculations. Surface-sterilized *G. tabacum* and *H. schachtii* were resuspended as above at a concentration of 50 J2/10 μL and 200 J2/10 μL for tobacco and *Arabidopsis* inoculations, respectively. Ten-microlitre aliquots of J2 were used to inoculate 14-day-old tobacco and 10-day-old *Arabidopsis* root tips grown in monoxenic culture as described previously. Penetration of roots by J2 was monitored using an inverted light microscope. Infected and noninfected transgenic root tissues were excised from Petri dishes at specific time points after penetration of roots by J2. For each time point examined, at least 100 infected and 30 noninfected transgenic roots were assayed for GUS expression using a compound photomicroscope.

For β-glucuronidase (GUS) assays, fresh, excised root pieces were infiltrated with GUS substrate (2 mM 5-bromo-4-chloro-3-indolyl beta-D-glucuronide [X-Gluc], 50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2% DMSO, 0.5 mM potassium ferrocyanide) and incubated overnight at 37 °C (Jefferson, 1987). To stain the nematodes, GUS-stained roots were placed in 1% hypochlorite for 4 min, washed in water for 10 min and then boiled for 2 min in a 1 : 10 diluted solution of acid fuchsin (3.5 g acid fuchsin, 250 mL acetic acid, 750 mL water). The roots were then rinsed in water and incubated in acidified glycerol for 2 days at 65 °C to clear the root tissue and reveal the stained nematodes (Hansen *et al.*, 1996). GUS-stained root pieces were placed in 70% ethanol for long-term storage. For sectioning of GUS-stained root pieces, the tissues were fixed in 4% paraformaldehyde for 3 days at 4 °C, washed in PBS, dehydrated in a graded ethanol series, incubated sequentially in Histoclear (National Diagnostics, Atlanta, GA): ethanol 25 : 75, 50 : 50, 75 : 25, and then in 100% Histoclear twice for 30 min each. The root pieces were incubated in Histoclear: Paraplast Plus (Fisher Scientific) 75 : 25 overnight at 60 °C, and then overnight again in pure Paraplast at 60 °C. The Paraplast-embedded root pieces were sectioned to a thickness of 15 μm (*Arabidopsis*) and 30 μm (tobacco) using a rotary microtome (American Optical, Buffalo, NY) and adhered to Superfrost Plus microscope slides (Fisher Scientific) overnight at 40 °C on a slide warmer. Three 15-min incubations in Histoclear were used to remove the Paraplast from sections, followed by rehydration in a graded ethanol series up to water prior to mounting with Permount (Fisher Scientific).

Tissues used for mRNA *in situ* hybridization were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated in an ethanol series and embedded in paraffin blocks as described above. Antisense and sense RNA probes were synthesized to the *UidA* (GUS) gene by *in vitro* transcription. Gene-specific primer sets containing *Eco*RI and *Bam*HI restriction sites were designed to the 3′ end of the *UidA* open reading frame (GenBank accession no. S69414) and used to amplify a 200-bp fragment. Primer sequences were as follows: UidA-3: 5′-GATCGCGTCAGCGCCGTCGTCG-3′ and UidA-4: 5′-GCAGCAGGGAGGCAAACAATGA-3′. The amplified product was digested with *Eco*RI and *Bam*HI and cloned into a pBluescript SK^+ transcription vector with a truncated multiple cloning site and flanking T3 and T7 promoter sequences (De Boer

et al., 1998). Purified plasmid DNA corresponding to the *UidA* riboprobe clone was digested separately with either *Eco*RI or *Bam*HI, column purified and 1–2 μg of linearized plasmid template was added to *in vitro* transcription reactions containing digoxigenin (DIG)–UTP (Roche Molecular, Indianapolis, IN) and the respective polymerase to synthesize RNA probes. Unincorporated nucleotides were removed using mini Quick Spin RNA columns (Roche Molecular) and the incorporation of DIG–UTP was quantified by dot blot analysis. *In situ* hybridizations of the *UidA* riboprobes to root-knot nematode-infected and noninfected *Cel1-GUS* tobacco root sections were conducted at 45 °C for 16–18 h followed by RNase A treatment (50 μg/mL) and two 1-h washes in 0.2× SSC at 55 °C. A final 1-h wash in 0.1× SSC was conducted at 55 °C prior to immunodetection of hybridized transcripts with sheep anti-DIG–alkaline phosphatase conjugate (Roche Molecular).

ACKNOWLEDGEMENTS

This research was supported by the North Carolina Agricultural Research Service, the North Carolina Tobacco Research Commission under grant 664855, and the North Carolina–Israel Partnership/ Binational Science Foundation under grant 1999011.

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