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Expression and Regulation of the *Arabidopsis thaliana Cel1* Endo 1,4 β Glucanase Gene During Compatible Plant-Nematode Interactions

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Abstract: The root-knot nematode *Meloidogyne incognita* is an obligate endoparasite of plant roots and stimulates elaborate modifications of selected root vascular cells to form giant cells for feeding. An *Arabidopsis thaliana* endoglucanase (*Atcel1*) promoter is activated in giant cells that were formed in *Atcel1*::*UidA* transgenic tobacco and *Arabidopsis* plants. Activity of the full-length *Atcel1* promoter was detected in root and shoot elongation zones and in the lateral root primordia. Different 5' and internal deletions of regions of the 1,673 bp *Atcel1* promoter were each fused to the *UidA* reporter gene and transformed in tobacco, and roots of the transformants were inoculated with *M. incognita* to assay for GUS expression in giant cells and noninfected plant tissues. Comparison of the *Atcel1* promoter deletion constructs showed that the region between −1,673 and −1,171 (fragment 1) was essential for *Atcel1* promoter activity in giant cells and roots. Fragment 1 alone, however, was not sufficient for *Atcel1* expression in giant cells or roots, suggesting that cis-acting elements in fragment 1 may function in consort with other elements within the *Atcel1* promoter. Root-knot nematodes and giant cells developed normally within roots of *Arabidopsis* that expressed a functional antisense construct to *Atcel1*, suggesting that a functional redundancy in endoglucanase activity may represent another level of regulatory control of cell wall-modifying activity within nematode feeding cells.
Key words: cellulase, cis-acting elements, giant cells, *Nicotiana tabacum*, parasitism, regulatory motif.

The root-knot nematode, *Meloidogyne incognita*, is an obligate endoparasite of plant roots that has evolved a complex feeding relationship with its host (reviewed in Davis et al., 2004). The structure of giant cells induced by root-knot nematodes includes extensive cell wall modifications acting as specialized feeding sites to allow cellular expansion and solute uptake (Jones and Northcot, 1972; Hussey and Grundler, 1998; Goellner et al., 2001; Gheysen and Fenoll, 2002; Vercauteren et al., 2002). The complex morphological and physiological changes during the establishment of giant cells and other nematode feeding sites (NFS) are reflected by altered gene expression in affected root cells (Wilson et al., 1994; Gheysen et al., 1996; Williamson and Hussey, 1996; Favery et al., 1998). The coordinated temporal expression and localization of cell wall-modifying enzymes that promote wall loosening and expansion in giant cells, and concomitantly promote cell-wall thickening and extensive ingrowths at the interface of neighboring vascular elements, likely represent augmentation of regulatory machinery active during normal plant cell wall growth and maturation.

The plant cell wall is a network of cellulose microfibrils, hemicellulose, pectin, and proteins that undergoes extensive changes in architecture during plant growth and development (reviewed in Carpita and Gibeaut, 1993). During the growth process, plant cells respond to multiple internal and external signals. In many cases, the response is translated into the loosening of the cell wall to enable turgor-driven cell expansion (Crosgrove, 1999; Rose and Bennett, 1999). Targeted enzymatic digestion of cellulose microfibrils by endogenous plant endoglucanases (EGases) is a tightly regulated process that is one primary component of cell-wall loosening (Fry, 1995; Rose and Bennett, 1999). The plant EGase genes identified to date are usually expressed within different developmental stages of the plant such as elongation, ripening and abscission (Lashbrook et al., 1994; Shani et al., 1997; del Campillo, 1999; Levy et al., 2002).

The *Arabidopsis thaliana* EGase gene, *Cel1* (*Atcel1*), is essential for normal plant cell growth and elongation, as it plays a role in cell wall deposition, cell differentiation, and cytogenesis (Tsabary et al., 2003). The primary activity of *Atcel1* has been observed during cell elongation and within fast growing tissues (Shani et al., 1997; Nicol et al., 1998; del Campillo, 1999; Shani et al., 2000). Overexpression of *Atcel1* resulted in accelerated growth of transgenic tobacco and poplar plants (Levy et al., 2002; Shani et al., 2004). Conversely, *A. thaliana* plants expressing *Atcel1* antisense exhibit shorter stems and roots, a corrugated cell wall surface, and fewer xylem elements per bundle, and both xylem elements and the interfascicular fibers are significantly less lignified than in the wild type (Tsabary et al., 2003).

Recently published evidence shows that elevated plant EGase activity localized in NFS may be, in part, responsible for the dramatic cell wall modifications observed within NFS (Goellner et al., 2001; Mitchum et al., 2004). Increased expression of five *Nicotiana tabacum* EGase (*Ntcel*) genes was detected within NFS induced in tobacco roots by both root-knot and cyst nematodes, and differential expression levels of each of

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the upregulated *Ntcel* genes in NFS were observed by semi-quantitative PCR (Goellner et al., 2001). The full-length promoter of *Atcel1* fused to GUS (Shani et al., 1997) was upregulated in giant cells induced by root-knot nematodes, but not within syncytia induced by cyst nematodes in either *N. tabacum* or *A. thaliana* host plants (Mitchum et al., 2004). The data suggest that differential expression of plant EGases gives rise to different NFS, but it is not clear how this activity may be regulated or which plant EGases are essential for proper formation of a given NFS. To this end, the activity of different deletions of the *Atcel1* promoter upon plant infection by root-knot nematodes and the response of plants expressing antisense to the *Atcel1* gene (Tsabary et al., 2003) to nematode infection have been investigated.

Materials and Methods

Atcel1 constructs and transgenic plants: Transgenic tobacco (*N. tabacum* SR1) and *A. thaliana* (Col-0 Ecotype) plants containing the full-length *Atcel1* promoter fused to the β-glucuronidase (GUS) reporter gene were developed previously (Shani et al., 1997; Tsabary, 2003). The construct was a transcriptional fusion between 1.6 kilobases (kb) of the putative Atcel1 promoter region (bases 5–1,618; Genbank accession X98543) and the 5' end of the β-glucuronidase gene (UidA) (Jefferson, 1987; Shani et al., 1997). The *Atcel1* promoter was divided into four 382 to 468 bp fragments (Fig. 1A), and six promoter::GUS constructs containing one to three fragments each were developed.

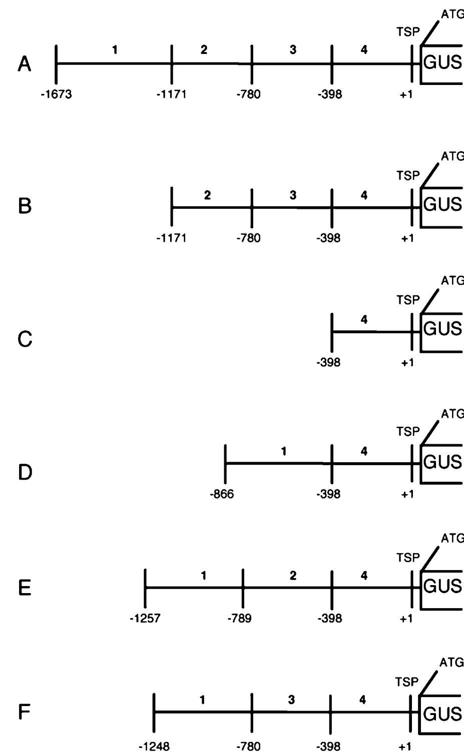

Fig. 1. Schematic representation of *Atcel1* promoter-*UidA* constructs in transformed tobacco plants analyzed for tissue expression and response to nematode infection. A) The full-length 1,673 bp *Atcel1* promoter, B-F) 5' *Atcel1* promoter deletion constructs harboring different lengths of the promoter (serial B and C, internal D, E, and F). Numbers indicate the length in bp of the respective promoter regions. Coding region of the β-glucuronidase gene. TSP transcription starting point, 1 to 4 indicate respective excised promoter regions.

To facilitate subcloning, each promoter segment was amplified using primers that contain restriction sites for the enzymes Hind III, Nde I and Sal I. The primers used were as follows: Fragment 1) 5'-AAAAAAGCTT-ACCTGCAGGTCAACGG-3' and 5'-AAAACATATGT-TCATTTAGTATATAACAAAATTCG-3'; Fragment 2) 5'-ATTTAAGCTTACACCATATGAAATGAACATTTG-CTCTGATTTGG-3 and 5'-AAAACATATGATTAT-TATATACTTTTTTTTTTATAAAAG-3'; Fragment 3) 5'-AAAAAAGCTTAAAACATATGTATATAATAATTT-ACACTCGAATC-3' and 5'- TGTGCATATGCTCAAT-AGTTGATTTTTGGAGG-3'; Fragment 4) 5'-AAAAAG-TTAAATCATATGGAGATCAAAACACGTGTCGC-3' and 5'-CCCCGTCGACGTCTCTTCTTTCTTGTGC-3'. The PCR reactions were performed using thermal cycling conditions of 94°C for 4 min, 30 cycles of 94°C for 10 sec, 55°C for 10 sec, and 72°C for 10 sec, and 72°C for 4 min using a buffer containing 1 Unit of DeepVent Taq polymerase (New England Biolabs, Inc., Beverly, MA), 20mM Tris-HCl pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 200μM dNTPs, 10 pmol primer and 1ng DNA template. The plasmid pUC18, containing a Sal I/Eco RI genomic fragment harboring the *At*cel1 promoter (Shani et al., 1997), was used as the template.

The fragments were purified, digested with different digestion enzymes (Fermentas Inc., Hanover, MD) and cloned, in various combinations, into pUC18 to create the promoter segment combinations shown in Figure 1. The promoter constructs were then digested with Hind III and Sal I and cloned into the binary vector pBinPlus, which contained the UidA.

The vectors were introduced into *Agrobacterium tumefaciens* using the freeze-thaw method (Erbert et al., 1988) and then transformed into tobacco. The constructs were then transformed into *N. tabaccum*-SR1 plants using the leaf-disc transformation as described previously (Horsch et al., 1985). Kanamycin-resistant plants were regenerated and confirmed by PCR. T2 homozygote plants were selected for further analysis.

Cel1 antisense *Arabidopsis* plants were previously developed and characterized by Tsabary et al. (2003). The construct contains bases 1 to 403 bp of the *Atcel1* coding region inserted in reverse orientation into the vector pBI101.1 containing the CaMV 35S RNA promoter and the octopine polyadenylation site (Tsabary et al., 2003).

Seeds of transgenic *Cel1-UidA* tobacco and *Arabidopsis*, *Cel1* antisense *Arabidopsis*, and nontransformed seeds were surface disinfected with 2.5% NaOCl and

0.5% sodium dodecyl sulfate (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/liter), and kanamycin (50 ug/ml). Tobacco and *Arabidopsis* seedlings were grown in a controlled temperature growth chamber at 25°C with a 16-hr photoperiod. At least five independent kanamycin-resistant lines were analyzed for each transgenic construct.

Nematode Infection: The root-knot nematode, *M. incognita*, was propagated on roots of greenhouse-grown tomato (*Lycopersicon esculentum* cv. Rutgers). *Meloidogyne incognita* eggs were isolated from egg masses on tomato roots with 0.5% NaOCl (Hussey and Barker, 1973), surface disinfected in a solution of 0.02% sodium azide for 30 min, rinsed with water on a 25-µm-opening sieve, and hatched in water at 28°C on a Baermann pan (Mitchum et al., 2004). Hatched *M. incognita* J2 were surface sterilized in 0.002% $HgCl_2$, 0.002% NaN_3, and 0.001% triton X-100 for 10 min, followed by five washes with sterile water. Surface-sterilized J2 were resuspended in 50 µl of 2 mM penicillin-G and 950 µl of 0.1% water agar immediately prior to inoculation of roots of plants grown on sterile nutrient agar. Five-microliter aliquots of *M. incognita* J2 were used to inoculate 10- to 12-day-old tobacco root tips and 10-day-old *Arabidopsis* root tips grown in monoxenic culture at a concentration of 15 J2/µl and 100 J2/5 µl, respectively. 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 all the time points examined, at least 100 infected and 30 uninfected transgenic roots were assayed for nematode infection and GUS expression. Promoter activity was also monitored in the root elongation zone and giant cells of control plants harboring the Δ0.6 TobRB7 promoter-UidA construct (Yamamoto et al., 1991) treated similarly as a positive control.

Histochemical GUS analysis: β-glucuronidase (GUS) activity was monitored by the method of Jefferson (1987) with some modifications (Yamaguchi et al., 2001). Fresh, excised root pieces were vacuum-infiltrated for 5 min with GUS substrate (2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide [X-Gluc], 100 mM Tris, pH 7.0, 50 mM NaCl, 0.06% Triton X-100) and incubated 12 hr at 37°C. In leaf samples excised to confirm construct (GUS) expression in experimental lines, chlorophyll pigmentation was removed by incubation of the samples for approximately 1 hr in 90% (v/v) ethanol. Samples stained for GUS activity were placed in 70% ethanol for long-term storage at 4°C.

Tobacco and *Arabidopsis* plants harboring the *Atcel:* GUS construct were analyzed for GUS expression following infection of host roots by *M. incognita* at 3, 4, 7, 14, 21 and 28 dpi in five to seven independent transformed lines for which 10 to 15 seedlings were assayed.

Histology of nematode feeding cells: Prior to sectioning, stained root pieces were fixed at 4°C in 4% paraformaldehyde for 16 hr for *Arabidopsis* and 3 d for tobacco, washed twice in PBS for 15 min after fixation, dehydrated in a graded ethanol series (30 min each), incubated sequentially in Histoclear (National Diagnosis, Atlanta, GA):ethanol 25:75, 50:50, 75:25, and then in 100% Histoclear twice for 30 min each time (Goellner et al., 2001). The root pieces were incubated in Histoclear:Paraplast Plus (Fisher Scientific) 75:25 overnight at 60°C and then overnight in pure Paraplast at 60°C. The Paraplast-embedded root pieces were sectioned to a thickness of 30 µm for tobacco and 10 µm for *Arabidopsis* using an 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 adhered to slides, followed by rehydration in a graded ethanol series to water prior to mounting with Permount (Fisher Scientific). For each time point, 15 to 30 infected roots were analyzed for GUS staining, and an equal number of uninoculated roots were analyzed for comparison.

Computational analyses of the Atcel1 promoter sequence: For putative motif analysis of the *Atcel1* promoter, we utilized the results of the Plant-CARE (Lescot et al., 2002) (http://oberon.fvms.ugent.be:8080/PlantCARE/index.html) and PLACE (Higo et al., 1999) (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) algorithms as described by Rombauts et al. (2003). To detect novel common regulatory elements in multiple promoters, the MOTIF SAMPLER algorithm (Thijs et al., 2001) was used (http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html). Consensus motifs identified using MOTIF SAMPLER were subsequently compared with the regulatory sites described in the Plant-CARE and PLACE databases.

Cel1 antisense A. thaliana: The development of *M. incognita* females in roots of *A. thaliana* expressing the antisense *Atcel1* construct (Tsabary et al., 2003) were compared to *M. incognita* development in roots of wild-type *A. thaliana*. Gross shoot and root morphology was compared to published descriptions to confirm the reported *Atcel1* phenotype (Tsabary et al., 2003) in test plants. The cellular morphology of infection sites in nematode-infected antisense roots as compared to wild-type was also evaluated using the fixation, embedding, and sectioning procedures described above. Sections were stained using Johansen's safranin/fast green protocol (Johansen, 1940) with some modifications (Ruzin, 1999) to enhance observable differences among cells and tissues. Photomicrographs of specimens were taken using a Nikon eclipse E600 microscope (Nikon

Instruments, Melville, NY) equipped with RT-color SPOT camera (Diagnostic Instruments, Sterling Heights, MI).

Results

Impact of Atcel1 promoter deletions on tissue-specific and nematode-induced expression: Tobacco plants transformed with the full-length or a truncated *Atcel1* promoter-*UidA* constructs were analyzed for promoter activity in uninfected plant tissue and within giant cells (Table 1). Five to seven independent transformed lines and at least 10 plants per transformed line were tested for each promoter construct. At least 100 tissue samples, including samples from each independent transformed line, were assayed for each promoter construct. Histochemical assays of β-glucuronidase (GUS) expression were used to analyze the temporal and spatial characteristics of the *Atcel1* promoter activity. During plant development, the expression of the full-length *Atcel1* promoter-*UidA* construct was observed in shoot and root elongation zones of infected and uninfected plants (Construct A, Figs.1,2A-B, Table 1). The expression of the full-length *Atcel1* promoter-*UidA* construct was not induced by mechanical wounding of the roots or leaves (data not shown). Low expression of construct B (Figs. 1,2C-D, Table 1), harboring a 502 bp 5′ deletion (promoter fragment 1), was observed in tobacco shoot elongation zones, but not in the roots. There was no obvious variation in GUS expression patterns among the transformed lines containing the same promoter construct, although slight variations in expression intensity could be observed. GUS activity was never observed in plants with constructs C to F, even though constructs D to F contained promoter fragment 1 (Fig. 1, Table 1). GUS activity was observed in the root elongation zone and giant cells of control plants harboring the Δ0.6 *TobRB7* promoter-*UidA* construct (Opperman et al., 1994), treated similarly as a positive control (data not shown).

We previously demonstrated that the full-length *Atcel1* promoter could drive *UidA* gene expression within giant-cells three days after nematode infection of *Arabidopsis* plants by root-knot nematodes (Mitchum et al., 2004). Upregulation of the full-length *Atcel1-UidA* construct (construct A) was observed within giant cells induced by root-knot nematodes in tobacco plants (Table 1, Fig. 2E-F) between 4 to 28 dpi. Similar results were obtained for all the time points used in this study. Frequently, full-length *Atcel1*-driven GUS activity was also visible early in the lateral root primordia, even in roots distant from the nematode feeding site. In contrast to full-length *Atcel1*, in all deletions in the *Atcel1* promoter assayed, including construct B, no detectable activity within the giant cells induced by *M. incognita* was observed (Fig. 1, Table 1).

Deletion of fragment 1 (−1,171 to −1,673) of the *Atcel1* promoter abolished expression in both giant cells and uninfected roots in all constructs examined. To determine whether all the motifs needed for *Atcel1* expres-

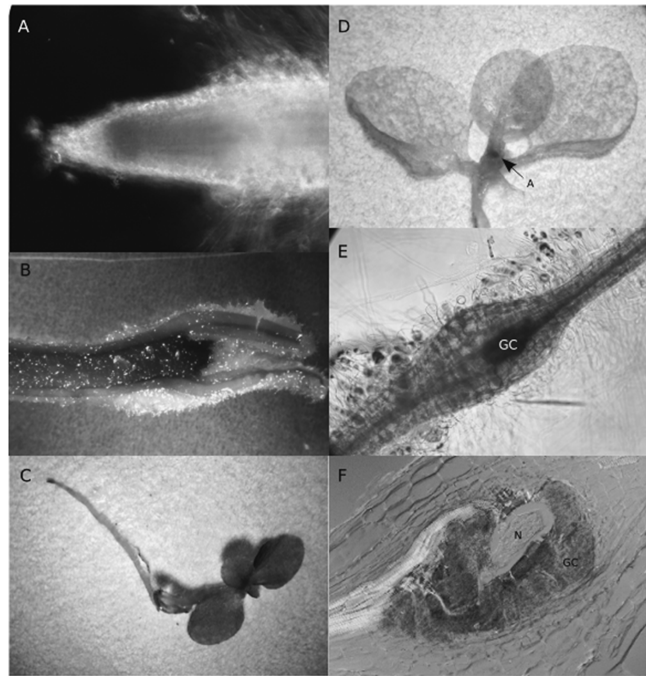

Fig. 2. Histochemical staining for GUS activity in transgenic tobacco plants containing the *Atcel1* promoter infected by the root-knot nematode, *Meloidogyne incognita*. A) *Atcel1*-driven GUS expression in a 7-day-old uninfected tobacco root. B) *Atcel1*-driven GUS expression in a shoot tip of a young uninfected tobacco seedling. C) *Atcel1* promoter deletion construct B (harboring a 502 bp 5' deletion, promoter fragment 1)-driven GUS expression in the shoot elongation zone of an uninfected tobacco plant. No activity is detectable in the roots. D) Whole-mount histochemical GUS assay of a *Cel1*-transgenic tobacco plant infected with *M. incognita*. *Atcel1* activity is confined to the nematode feeding cells (not shown) and the plant elongation/differentiation zones. A = shoot meristem (Construct B shown). E) *Atcel1*-driven GUS expression within *M. incognita*-induced giant cells four days post-inoculation of nematodes to an *Atcel1-GUS* transgenic tobacco root (construct A, Fig. 1). GUS activity is confined to the central region of the developing gall tissue. F) Sections (30 μm thick) through *M. incognita*-infected *Atcel1-GUS* tobacco roots after GUS staining. GUS expression is restricted to the giant cells induced by the nematode. N = nematode, GC = giant-cells,

Table 1. Activity of the *Atcel1* promoter-*UidA* constructs in roots, shoot, leaves and root-knot nematode feeding sites (NFS).

Construct[a]	GUS Expression[b]			No. of GUS + NFS/ total no. NFS
	Root	Shoot/leaves	NFS	
A	+	+	+	175/205
B	−	+	−	0/313
C	−	−	−	0/258
D	−	−	−	0/136
E	−	−	−	0/147
F	−	−	−	0/100

[a] A denotes the full-length 1,673 bp *Atcel1* promoter; B–F are 5′ *Atcel1* promoter deletion constructs (serial B and C, internal D, E, and F).

[b] The activity of the reporter gene in roots, shoots, leaves and in NFS as visually determinate are indicated as follows: + and − indicate presence or absence of GUS activity, respectively. Number of nematode feeding sites were collected between 3 to 28 dpi. Similar results were obtained for all the time points used in this study.

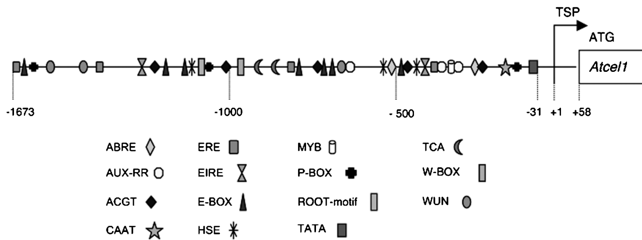

FIG. 3. Putative cis-acting elements of the *Atcel1* promoter as predicted by the Plant-CARE (Lescot et al., 2002), PLACE (Higo et al., 1999; Rombauts et al., 2003), and MOTIF SAMPLER (Thijs et al., 2001) algorithms. The transcription start point (TSP) is indicated with +1: transcription start point. Distances in bp are relative to the translation start codon.

sion in roots and in NFS were in fragment 1 of the *Atcel1* promoter (Fig. 1), several internal deletions between fragments 1 and 4 were examined (Fig. 1D-F; Table 1). No internal *Atcel1* promoter deletions that included fragment 1 were sufficient for GUS expression in NFS or roots.

Identification of conserved sequence motifs in the Atcel1 promoter: Because the *Atcel1* promoter is upregulated within giant cells induced by root-knot nematode, we analyzed the promoter for specific sequences that act as nematode responsive cis-acting elements that could be responsible for the observed GUS gene expression. Plant-CARE, PLACE, and MOTIF SAMPLER analyses of the 1,673 bp *Atcel1* promoter sequence revealed a number of predicted functional motifs found in most eukaryotic promoters, in addition to several potential regulatory elements that have been shown to be functional in other plant promoters (Fig. 3). A typical TATA box was identified at position −31, and a CAAT box-like sequence was found at position −50 relative to its transcription start point (TSP), respectively. The upstream sequence relative to the TSP of the Atcel1 has several regions with over 80% A/T content (data not shown).

To evaluate sequence motifs that may be common among promoters of NFS-expressed genes, we compared motifs found in the *Atcel1* promoter to those of other genes known to be upregulated in NFS. Included were characterized promoters of five other *Arabidopsis* genes, in addition to *Atcel1*, that are known to be upregulated in NFS, as well as three NFS-responsive promoters from other plant species (Table 2). In particular, the 300 bp 'nematode box' from the tobacco *TobRB7* promoter and a 246 bp fragment from the *Hahsp17.7G4* sunflower promoter were included because functional studies have definitively linked these minimal nematode-inducible sequences with NFS-specific expression. Two *Arabidopsis* promoters reported to be down regulated in NFS were included as negative controls.

Analysis of the promoter regions using MOTIF SAMPLER revealed the presence of several putative regulatory elements that were previously reported in NFS upregulated genes, including E-BOX, AUX-RR, ROOT-MOTIF, and W-BOX (Fenoll et al., 1997; Escobar et al., 1999; Puzio et al., 2000; Mazarei et al., 2002; Thurau et al., 2003). All of these sequences were present in one or both of the promoter sequences from the NFS downregulated genes, suggesting that they do not, in fact, represent NFS-specific transcription factor binding sites. Four motifs (EIRE, ERE, P-BOX, and WUN-MOTIF) were present in the NFS upregulated promoter sequences but not in the downregulated ones (Table 2).

Nematode infection of antisense Atcel1 plants: To further investigate the potential function of *Atcel1* during root-knot nematode infection, three *Arabidopsis* lines expressing an antisense *Cel1* construct (Tsabary et al., 2003) were infected with root-knot nematode. Samples of infected and uninfected plant roots were collected at 3, 4, 7, 14, 21 and 28 dpi. Samples were fixed, sectioned,

TABLE 2. Putative regulatory elements common between the *Atcel1* promoter (accession X98543) and other plant promoters activated and downregulated in feeding cells induced by root-knot nematodes (RKN) and cyst nematodes (CN).

			Element[a]						
Gene construct[c]	Accession	Induced by	E-BOX CANNTG	EIRE TTCGacc	ERE ATTTcaaa	P-BOX CCTTtg	W-BOX[b] TTGACC	WUN-MOTIF aAATTtcct	Reference
ATCEL1-FRAG1-GUS	X98543	RKN	X	X	X	X	X	X	Mitchum et al., 2004
ATPYK20-GUS	AJ249204	RKN, CN	X	X	X	X	X	X	Puzio et al., 2000
AT#25.1-GUS	A91914	RKN,TCN	X	X	X	X	X	X	Ohl et al., 1997
AT#1164-GUS	A79355	RKN, CN	X		X		X		Ohl et al., 1997
ATSUC2-GUS	X79702	RKN, CN	X		X	X	X	X	Juergensen et al., 2003
ATATAO1-GUS[c]	AF034579	RKN, CN	X					X	Moller et al., 1998
LELEMMI9-GUS	S45406	RKN	X	X	X	X	x	X	Escobar et al., 1999
NTTOBRB7-0.3-GUS	S45406	RKN	X		X			X	Opperman et al., 1994
HAHSP17.7G4_83-GUS	U46545	RKN		X				X	Escobar et al., 2003
ATPAL1-GUS[d]	X62747	RKN, CN	X				X		Goddijin et al., 1993
AT-TIP-GUS[d]	X63552	RKN, CN							Goddijin et al., 1993

[a] Regulatory motifs predicted by the Plant-CARE (Lescot et al., 2002), PLACE (Higo et al., 1999; Rombauts et al., 2003), and MOTIF SAMPLER (Thijs et al., 2001) algorithms.
[b] Motif was present in the *ATCEL1* promoter but not in the ATCEL1-FRAG.1.
[c] *AT: Arabidopsis thaliana, LE: Lycopersicon esculentum, NT: Nicotiana tabacum.*
[d] Gene constructs that are downregulated in response to nematode infection.

stained, and examined for general root morphology as well as the ability for *M. incognita* to induce giant-cell development. Uninfected *A. thaliana* plants containing the *Atcel1* antisense construct exhibited the same alterations in shoot and root morphology as previously reported (Tsabary et al., 2003). The *Atcel1* antisense plants had shorter stems and roots relative to the wild-type plants (Fig. 4A), indicating that the antisense construct was actively expressed.

Microscopic examination of stained root sections revealed a typical pattern of *M. incognita* infection and development, as well as typical giant cell formation (Fig. 4B,C). Similar patterns of nematode and giant-cell development were observed in both the wild-type and in the *Atcel1* antisense constructs. Similar numbers of NFS developed in wild-type and in the *Atcel1* antisense infected plants (1,151 galls/50 plants in wild type vs. 1,185 galls/50 plants in antisense plants; data from two repetitions). Because sections from both wild-type *Arabidopsis* (Fig. 4B) and *Atcel1* antisense *Arabidopsis* (Fig. 4C) revealed that *M. incognita* J2 penetrated roots of both plant types equally and that giant cell and nematode development were comparable, we did not count numbers of adult female nematodes. However, we did observe that the nematodes completed their life cycle in the antisense plants.

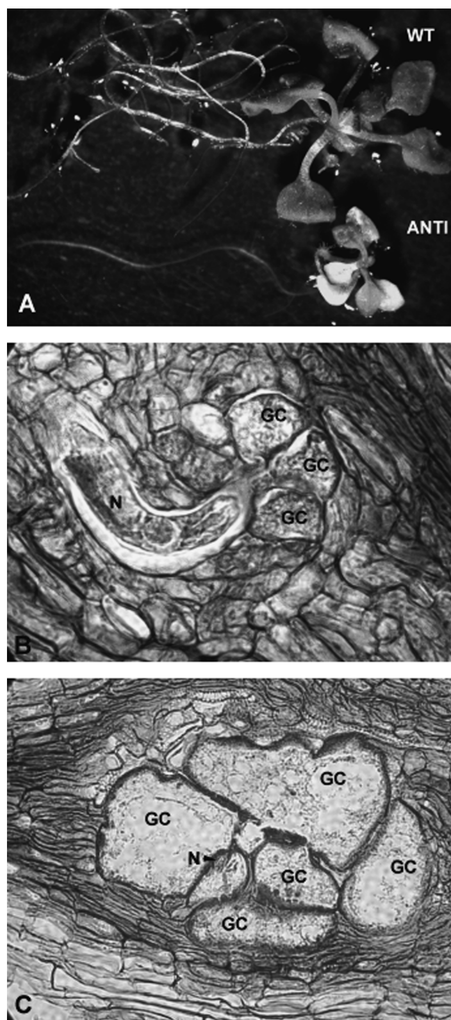

Fig. 4. Constitutive expression of antisense *Atcel1* in transgenic *A. thaliana* and infection of these plant roots with the root-knot nematode, *M. incognita*. A) Both shoot and root development of *Atcel1* antisense (ANTI) plants are compromised as compared to wild-type (WT) *Arabidopsis*. B) Giant cells (GC) form normally around the head of a developing root-knot nematode (N) in a (10-µm-thick) cross-section of a wild-type *Arabidopsis* root. C) Giant cell and nematode development in antisense *Atcel1 Arabidopsis* progress normally even as root and shoot development are compromised. Abbreviations are defined in the text and in Table 2.

Discussion

The observed upregulation of the *Atcel1* promoter within giant cells induced in roots by root-knot nematodes and the lack of this activity within the feeding sites of cyst nematodes suggested potential transcriptional regulation of *Atcel1* expression upon nematode infection (Mitchum et al., 2004). In this study, a comparative analysis of *Atcel1* promoter deletion constructs demonstrates that the region between −1,673 and −1,171 (fragment 1) was essential to provide specificity of *Atcel1* promoter expression in roots and giant cells. It is unclear if elements within fragment 1 of the *Atcel1* promoter that are required for expression within roots are also required for expression within giant cells. Some analyses of gene expression within giant cells (Wilson et al., 1994; Gheysen and Fenoll, 2002) indicate that wild-type expression in roots was not a prerequisite for plant genes recruited during giant-cell formation. The expression of *Atcel1* promoter construct B (containing a deletion of fragment 1) in shoots observed here, however, was uncoupled from expression in giant cells and roots. It has been demonstrated with promoter deletions of the *TobRB7* gene of tobacco that elements that drive root-specific expression can be uncoupled from elements that can drive expression specifically within giant cells (Opperman et al., 1994). Similarly, the −83 to +163 region of the *Hahsp17.7G4* gene in sunflower that contains heat shock element core sequences was sufficient to drive expression within giant cells (Escobar et al., 2003). Inclusion of fragment 1 of the *Atcel1* promoter in the absence of internal regions within the *Atcel1* promoter constructs analyzed here, however, indicates that the presence of a fragment 1 alone within *Atcel1* is not sufficient to drive expression within giant cells. The effects may be due simply to the relative change in distance and conformation between upstream and downstream elements. In general, the function of a regulatory region is complex, involving a multiprotein complex interacting with the transcription factors bound to neighboring DNA sites. A supplementary layer of complexity is added by bringing the transcription factors together on the promoter and by adopting a three-dimensional configuration, enabling the interaction with other parts to activate the

basal transcription machinery (Buratowski, 2000; Rombauts et al., 2003).

Alternatively, the activity of other functional elements within the *Atcel1* promoter may also be required for both the root and giant-cell expression. A number of conserved sequence motifs that may represent transcription factor binding sites were found within the *Atcel1* promoter as well as in promoter regions of other nematode induced genes. Interestingly, these motifs include WUN-motif (Van de Loecht et al., 1990; Washida, et al., 1999) and EIRE (Shah and Klessig, 1996; Fuduka, 1997), both of which have been reported in genes that are transcriptionally activated in response to pathogen-derived elicitors. The ethylene responsive element (ERE) is found in many pathogenesis-related (PR) genes that are activated during nematode infection (Schwechheimer et al., 1998; Mazarei et al., 2002). The ERE motif was also found in within the *Atcel1* promoter, but not in the promoter regions of nematode-repressed genes. Whether these motifs have specific roles in transcriptional modulation of *Atcel1* remains to be determined; however, they represent candidates for further functional analyses using directed mutagenesis and/or deletions.

Regulation of plant endoglucanase expression at the transcriptional level may be only one level of control of cell wall-modifying activity induced by nematodes within feeding cells. The inability of a functional *Atcel1* antisense to affect giant-cell or nematode development here suggests that *Atcel1* activity may not be essential for proper giant-cell formation and/or that functional redundancy in induced endoglucanase activity within NFS exists. In *Arabidopsis*, the EGase gene family comprises more than 20 members (del Campillo, 1999; Tsabary et al., 2003). Potential functional redundancy is supported by the upregulation of at least five tobacco endoglucanase genes within the feeding cells of both root-knot and cyst nematodes (Goellner et al., 2001). The tobacco endoglucanases upregulated in NFS are phylogenetically distinct, however, and may represent functional differences in both normal plant development and activity within NFS. It remains to be investigated whether an endoglucanase essential to the formation of NFS can be identified.

These results indicate that expression of the *Atcel1* promoter in NFS is regulated by the combinatorial interactions of cis-acting regulatory elements in the promoter, including essential element in the distal region of the promoter. The multiple putative cis-acting elements (Fig. 3) of the *Atcel1* promoter accommodate the argument that they may act as coupling elements that may function in different combinations to confer a diversity of tissue-specific, developmental, and stress-regulated patterns. The promoter deletions examined did not result in restricting activity to giant cells as observed with the Δ0.3 *TobRB7* element (Opperman et al., 1994). Further work, including the generation of a fine-scale series of 5' promoter deletions within fragment 1 in combination with linker scanning and or/site-directed mutagenesis will be required to precisely define, if possible, given cis-elements within the *Atcel1* promoter that convey a specific response in NFS. This potential has important implications for strategies to engineer nematode resistance. Nematode-responsive promoters may be used to localize the expression of anti-nematode constructs that interfere with the development of the feeding site and/or nematode specifically to nematode infection sites (Atkinson, 2003). When targeting plant endoglucanase genes for inhibition within NFS, one must consider potential functional redundancy of endoglucanase activity within NFS as well as whether the target gene is essential to the success of NFS formation.

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