

Isolation of Beta-1,4-Endoglucanase Genes from *Globodera tabacum* and their Expression During Parasitism¹

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Abstract: Two beta-1,4-endoglucanase (EGase) cDNAs were isolated from *Globodera tabacum*, the tobacco cyst nematode, and have been designated as GT-*eng-1* and GT-*eng-2*. GT-*eng-1* and GT-*eng-2* encode precursor proteins with a predicted secretion signal sequence, cellulolytic catalytic domain, and a linker domain. The protein product GT-ENG-1 contains an additional 95 amino acid carboxy terminal sequence with strong similarity to type II cellulose binding domains. Riboprobes and polyclonal antibodies raised to recombinant cyst nematode EGases were used to follow expression patterns of EGase transcripts and proteins throughout the nematode life cycle. EGase transcripts and proteins were specifically detected within the subventral esophageal gland cells of *G. tabacum* second-stage juveniles (J2) within eggs prior to hatching, in preparasitic J2, and in parasitic J2 that had invaded tobacco roots. EGase transcripts and proteins were not detected in *G. tabacum* after the molt to the sedentary J3, J4, and adult female life stages. Interestingly, EGase transcription and translation resumed in the subventral esophageal glands of late J4 males. It is hypothesized that secreted EGases play a major role to facilitate intracellular migration of *G. tabacum* within tobacco roots.

Key words: beta-1,4-endoglucanases, EGase, esophageal gland cells, *Globodera tabacum*, *Nicotiana tabacum*, stylet secretions, tobacco, tobacco cyst nematode.

Beta-1,4-endoglucanases (EGases) are a class of hydrolytic enzymes produced by a diverse group of organisms including bacteria, fungi, insects, and plants (Brummel et al., 1994; Davis et al., 2000; Henrissat and Bairoch, 1993). EGases function in the hydrolysis of the beta-1,4-glucan backbone of polymers such as cellulose and xyloglucan, which are two of the major carbohydrate components of the plant cell wall (McNeil et al., 1984). An ensemble of endogenous hydrolytic enzymes, including EGases, promotes the degradation of cell walls to serve as a primary carbon source for many microorganisms (Beguin and Aubert, 1994). Additionally, cellulolytic enzymes function as pathogenicity factors for many phytopathogenic fungi and bacteria by facilitating entry into host tissue and enhancing access to

plant cell contents (Barras et al., 1994; Roberts et al., 1988).

Until recently, no direct evidence existed for the endogenous production of cellulolytic enzymes in animals. Smant et al. (1998) isolated and cloned the first EGases of animal origin from the plant-parasitic cyst nematodes *Globodera rostochiensis* and *Heterodera glycines*. This discovery is the first direct evidence in support of earlier biochemical studies that suggested that nematodes produced cellulolytic enzymes (Bird et al., 1975; Deubert and Rohde, 1971). Genes for EGases with and without a predicted cellulose-binding domain (CBD) were cloned from both species of cyst nematode and named *eng-1* and *eng-2*, respectively (Smant et al., 1998). It was demonstrated that the EGases are produced endogenously within the two subventral esophageal gland (SvG) cells of cyst nematodes and are secreted through the nematode stylet into plant tissue (De Boer et al., 1999; Smant et al., 1997; Smant et al., 1998; Wang et al., 1999). Expression of EGases in the SvG of *H. glycines* ends in the early parasitic J3 stage and, interestingly, resumes only in males as they develop to adults (De Boer et al., 1999). It is hypothesized that these enzymes may facilitate penetration and intracellular migration of root tissue by nematodes during parasit-

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ism. In addition, cyst nematode EGases could contribute to the partial cell wall dissolution that occurs among neighboring cells of developing feeding sites (syncytia; Jones and Dropkin, 1975; Jones, 1981), although this has not been definitively demonstrated.

The monoclonal antibody (MGR48) used to immunopurify EGases from *G. rostochiensis*, and *H. glycines* (Smant et al., 1988) also recognized SvG antigens of other cyst nematode species including *G. tabacum*, the tobacco cyst nematode (De Boer et al., 1996). *Globodera tabacum* is a sedentary endoparasite with a host range limited to tobacco and some solanaceous weeds (Miller and Gray, 1972). If cellulase production is a common mechanism used by plant-parasitic nematodes to invade roots and to establish feeding sites, then this may be a feasible target for developing transgenic strategies to improve plant disease resistance. The *G. tabacum*-tobacco interaction provides a genetically amenable model system, which can be used to test transgenic approaches that target nematode cellulases for resistance to plant-parasitic nematodes. In this paper, we describe the isolation of two *G. tabacum* beta-1,4-endoglucanase cDNAs, *GT-eng-1* and *GT-eng-2*, and monitor their expression during the parasitic cycle.

MATERIALS AND METHODS

Nematode cultures: *Globodera tabacum* subspecies *solanacearum* (Miller and Gray, 1972) was propagated on greenhouse-grown tobacco (*Nicotiana tabacum* 'NC95'). Cysts of *G. tabacum* were extracted from infested soil by flotation in water and collected on a 250- μ m sieve. The cysts were transferred to centrifuge tubes and mixed with 70% (w/v) sucrose in a 1:1 ratio. Cysts were separated from contaminating debris by centrifugal flotation at 1,000g for 5 minutes. Subsequently, the cysts were gently crushed in a glass homogenizer to release nematode eggs, which were collected on a 25- μ m sieve. To collect preparasitic second-stage juveniles (J2), eggs were hatched over water at 28 °C on a Baermann pan. Some aliquots of

fresh J2 and other life stages of *G. tabacum* were used for protein and nucleic acid extractions, and some were fixed for localization analyses.

For in situ hybridization and immunolocalization analyses, eggs were fixed in 2% paraformaldehyde in M9 buffer (42 mM Na₂HPO₄, 22 mM KH₂PO₄, 85 mM NaCl, 1 mM MgSO₄ · 7H₂O, pH 7.0) plus 10% sucrose for 5 days at 4 °C. Preparasitic J2 were fixed in 2% paraformaldehyde in M9 buffer overnight at 4 °C. Mixed parasitic stages of *G. tabacum* were collected from infected tobacco roots that were washed, cut into 2.5-cm pieces, suspended in water, and chopped in a blender for 30 seconds. An equal volume of 2× fixative (4% paraformaldehyde in 2× M9 buffer) was added to the root suspension and incubated at room temperature for 1 hour for initial fixation to reduce mRNA degradation. The root suspension was poured over a stack of sieves, and parasitic nematodes were collected on a 25- μ m sieve and rinsed in water. Fixed parasitic nematodes were separated from root debris by mixing the slurry 1:1 with 70% sucrose and overlaid with a 1/4 volume of water. The sucrose suspension was centrifuged at 1,000g for 5 minutes, nematodes were pipetted from the upper 2/3 of the suspension, and rinsed with water. Distinct nematode developmental stages were identified under a dissecting microscope, separated by hand, and fixed further in 2% paraformaldehyde in M9 buffer. Parasitic J2 and adult males were fixed overnight at 4 °C in 2% paraformaldehyde in M9 buffer. All other parasitic life stages were fixed overnight at room temperature. Fixed nematodes were collected, rinsed in M9 buffer, and stored at -80 °C.

Isolation of Globodera tabacum EGase cDNA's: Total RNA was isolated from preparasitic J2 using the Pharmacia Biotech (Piscataway, NJ) Cs-TFA RNA extraction kit according to the manufacturer's protocol. *Globodera tabacum* EGase cDNAs were amplified using 3' and 5' random amplification of cDNA ends (RACE) PCR systems (GibCo BRL, Rockville, MD). *Globodera tabacum* mRNA was converted into cDNA using reverse transcriptase

and the supplied oligo-dT primer. *Globodera rostochiensis* EGase primers (Smant et al., 1998) employed in 3' RACE (CELSIG1.2) and 5' RACE (GSVPUP and SVP39-INT1) to amplify *G. tabacum* sequences were as follows (Fig. 1): CELSIG1.2, 5'-CTTCCGTGTCTTCCTCCTCCATG-3'; GSVPUP, 5'-CCAATTGCATTTGAGYGCCTTCACTG-3'; SVP39-INT1, 5'-TGIGTRTCIACDATNACRTACATKCC-3' (I = inosine, Y = C + T, R = A + G, D = G + A + T, N = A + C + G + T, K = G + T). Products were cloned into the pCR2.1 TA vector (Invitrogen, Carlsbad, CA), and the *G. tabacum* cDNA clones were sequenced by The Interdisciplinary Center for Biotechnology Research (ICBR) DNA Sequencing Core Laboratory (DSEQ) located at the University of Florida, Gainesville, Florida.

Digoxigenin (DIG)-labeled riboprobe: Sequences corresponding to the 5' end of *GT-eng-2* (nucleotides 1-263) and the cellulose-binding domain (CBD) of *GT-eng-1* (nucleotides 1179-1373) were subcloned into a pBluescript II KS transcription vector with a shortened multiple cloning site (De Boer et al., 1998). The *GT-eng-1* CBD riboprobe spanned nucleotide sequence that was not present in *GT-eng-2*, making it a gene-specific probe (Fig. 1). The high-percentage

nucleotide sequence identity between *GT-eng-1* and *GT-eng-2* did not allow for the construction of a riboprobe specific to *GT-eng-2*; thus, the *GT-eng-2* riboprobe hybridized with both transcripts. Plasmid DNA was isolated using the Wizard Plus Miniprep DNA purification system (Promega, Madison, WI) and digested with restriction enzymes flanking the *GT-eng* insert. Linearized plasmid samples were extracted with phenol:chloroform, precipitated, and resuspended in TE buffer at a concentration of 0.5 to 1.0 µg/µl. Digoxigenin-labeled antisense and sense RNA probes were transcribed in vitro using either T7 or T3 polymerases as described by De Boer et al. (1998).

In situ hybridization: Fixed *G. tabacum* eggs, preparasitic J2, and parasitic J2 were suspended in diluted fixative and cut into pieces on a glass slide using a single-edge razor blade attached to a vibrating aquarium air pump (De Boer et al., 1998). Juveniles of the J3 and J4 stages as well as adult males and females were cut manually under a dissecting microscope with a scalpel. Nematode sections were treated with Proteinase K, methanol, and acetone; rehydrated; and rinsed in prehybridization solution as described by De Boer et al. (1998). Processed nematode sections were prehybridized at

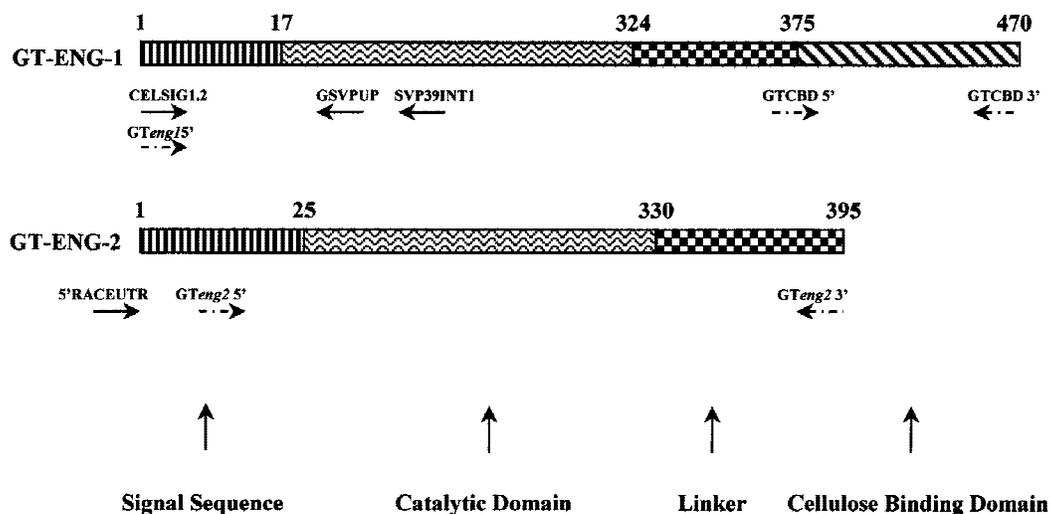


FIG. 1. Predicted protein domains of *Globodera tabacum* beta-1,4-endoglucanases (*GT-ENG-1* and *GT-ENG-2*) and positions of PCR primers used for gene cloning (straight arrows) and recombinant protein expression (dotted arrows).

42 °C (GT-*eng-2* riboprobe) or 48 °C (GT-*eng-1* CBD riboprobe) for 30 minutes, then DIG-labeled riboprobe was added to a final dilution of 1:2000. Probes were hybridized overnight at the respective prehybridization temperature. Post-hybridization treatments of the nematode sections were as described by De Boer et al. (1998). Hybridized probe was visualized by incubation of nematode sections in alkaline phosphatase-conjugated sheep anti-DIG Fab antibody fragments and subsequent color reaction with bromochloro-indolyl phosphate and nitroblue tetrazolium (De Boer et al., 1998). Color development was stopped by rinsing nematodes with water prior to mounting on slides for observation using differential interference contrast microscopy.

Carboxymethylcellulase assay: Total protein of *G. tabacum* parasitic J2 was isolated by grinding nematodes in 62.5 mM Tris pH 6.8 amended with protease inhibitors (10 µl/ml of Stock A = 0.1 mM leupeptin, 100 mM Na₂EDTA, 20 mM iodoacetamide and Stock B = 20 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin A [all chemicals from Sigma, St. Louis, MO]). Nematode proteins were separated by partially denaturing polyacrylamide gel electrophoresis (PAGE) run under partial-denaturation conditions; SDS was added at 0.1% final concentration to the electrode buffer, and 0.1% high-viscosity carboxymethylcellulose (CMC) was added into the polyacrylamide gel. The gel was incubated for 30 minutes in three changes of 2% Triton X-100 to remove SDS and to enable the proteins to renature. The gel was then washed twice for 5 minutes each with 50 mM sodium phosphate buffer, pH 5.5 to remove Triton X-100. The gel was incubated in 50 mM sodium phosphate buffer, pH 5.5 at 37 °C for 17 hours followed by staining with aqueous 0.1% Congo red solution for 30 minutes (Teeri, 1997). The gel was rinsed with 1 M NaCl, and cleared zones indicated protein bands where the CMC substrate was hydrolyzed by endoglucanase activity.

Expression of *G. tabacum* EGases in *E. coli*: Primers specific to either GT-*eng-1* or GT-*eng-2* (containing Bam HI and Xho I, or Bam HI and Hind III restriction sites) were

used to amplify and subsequently clone each predicted full-length open reading frame into the pET28c expression vector that incorporates a His-tag for affinity isolation of recombinant proteins (Novagen, Madison, WI). GT-*eng-1* primers were as follows (Fig. 1): GT-*eng1-5'*; 5'-CGCGGATCCGCTTCCGTGTCTTCCTCCTC-3' and GTCBD-3'; 5'-CCGCTCGAGTCGCCCCGAACATGCCGT-TGT-3'. GT-*eng-2* primers were designed as follows: GT-*eng2-5'*, 5'-CGCGGATCCAAATGTGTCGTCTCCAGTCC-3' and GT-*eng2-3'*; 5'-CCCAAGCTTGGGTCTTAGCCC-GATTTGGAC-3'. Recombinant proteins were overexpressed in *Escherichia coli* host strain BL21(DE3) (Novagen, Madison, WI) according to the manufacturer's guidelines. Expressed proteins contained in inclusion bodies were solubilized in 6 M Urea and purified over a nickel affinity column. Purified recombinant proteins were dialyzed in buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) and tested for cellulolytic activity by spotting approximately 0.5 µg onto 0.2% CMC agarose plates, incubating for 3 hours at room temperature, and staining with 0.1% Congo red solution.

Production of GT-ENG-1 specific antisera: Polyclonal antibodies to the *G. rostochiensis* ENG-1 and ENG-2 proteins were previously prepared to the full-length recombinant proteins (Smant et al., 1998). Both *G. rostochiensis* polyclonal antibodies cross-reacted with both *G. tabacum* EGases. The high-percentage amino acid sequence identity between regions of either *G. rostochiensis* or *G. tabacum* ENG-1 and ENG-2 did not allow for the production of antisera specific to ENG-2. To make antisera that would react only with GT-ENG-1, primers specific for the GT-*eng-1* CBD (absent in GT-ENG-2) were designed with Bam HI and Xho I restriction sites for amplification and subsequent subcloning of a 335-basepair (bp) fragment (spanning amino acid's 360–470) into the pET28c expression vector. Primers were as follows (Fig. 1): GTCBD-5'; 5'-CGCGGATCCCTTCGTCTTCGCGGGGTC-3' and the GTCBD-3' primer. With the additional amino acids from the expression vector, the recombinant protein was predicted to be ap-

proximately 20 kD. The recombinant protein expressed within inclusion bodies was solubilized with 6 M urea, recovered over a nickel affinity column, and further purified by SDS-PAGE. Gel slices containing approximately 100 µg of protein were ground to a fine powder in liquid nitrogen, resuspended in a small volume of buffer, and mixed 1:1 with Freud's complete adjuvant for the first injection of New Zealand white rabbits. Two subsequent injections were given as above, with Freud's incomplete adjuvant at 2-week intervals. All injections were subcutaneous, and antisera was collected 2 weeks after the final immunization.

Immunolocalization: Immunolocalization procedures were identical to the in situ hybridization protocol up to the prehybridization step. Instead of washing with prehybridization solution, sections of different nematode developmental stages were rinsed with phosphate-buffered saline (137 mM NaCl, 1.4 mM KH₂PO₄, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, pH 7.4) containing 0.5% Triton X-100 [PBST]). The nematodes then were incubated in 10% goat serum (Sigma, St. Louis, MO) in phosphate-buffered saline amended with protease inhibitors (as described above) overnight at room temperature on a rotator. GR-ENG-1 antisera was added to a 1:300 dilution, and the nematodes were incubated overnight at room temperature on a rotator. GT-ENG-1 CBD antiserum was assayed at a 1:100 dilution in nematode specimens (even though negative). Following incubation, the nematodes were washed three times for 5 minutes each with PBST. Nematodes were incubated in a 1:500 dilution of goat anti-mouse FITC-conjugated secondary antibody (Sigma, St. Louis, MO) in Tris-Saline-BSA (0.15 M NaCl, 0.01 M Tris, pH 7.2, 0.2% Triton X-100, 3% BSA) for 3 hours at room temperature on a rotator. Nematodes were washed twice in PBST and once in distilled water prior to mounting on slides. Nematodes were observed on an epifluorescence microscope (Zeiss, Oberkochen, Germany).

Induction and collection of cyst nematode stylet secretions: Freshly hatched J2 of *G. tabacum* were collected and pelleted in 50-ml conical

plastic centrifuge tubes at 1,000g, rinsed twice with water, and resuspended in 12 ml of either the serotonin receptor agonist 5-methoxy-N,N-dimethyltryptamine hydrogen oxalate (DMT; RBI Inc., Natick, MA) at 0.3 mg/ml in distilled water to induce secretions from the J2 stylet (Goverse et al., 1994) or in distilled water (non-induced control). J2 in DMT or water were incubated in small glass petri dishes at 28 °C for 4 to 8 hours. Coomassie brilliant blue G-250 was added to a subsample of DMT-treated J2 to monitor the production of stylet secretions. The secretions were solubilized by adding an equal volume of 0.1 M Tris-NaOH, pH 11.0. The suspension was transferred to a 15-ml corex tube, and the worms were pelleted at 12,000 rpm at 10 °C for 3 minutes. Solubilized secretions in the supernatant were concentrated using 10,000 MWCO Centricon concentrators (Amicon, Beverly, MA). Concentrated stylet secretions were pooled over a 7-day period and then further concentrated to a final volume of 25 µl using a 10,000 MWCO Microcon concentrator (Amicon, Beverly, MA).

Western blots: Concentrated stylet secretions were separated by SDS-PAGE, electroblotted to Immobilon-P membranes (Millipore, Bedford, MA), and probed with either GR-ENG-1 antisera (1:1000) or GT-ENG-1 CBD antisera (1:5000) using standard protocols with alkaline phosphatase-conjugated secondary antibody (Towbin et al., 1979). Total protein of *G. tabacum* developmental stages (collected as described above, but with omission of the nematode fixation step) was isolated by grinding nematodes in 200-µl glass homogenizers in 62.5 mM Tris-HCl pH 6.8 amended with protease inhibitors (as described above). The homogenate was centrifuged at 12,000g for 15 minutes at 4 °C to remove insoluble particulate matter. Protein samples were quantified according to Bradford (1976), using bovine serum albumin as a standard. Fifteen micrograms of total protein from each life stage was separated by SDS-PAGE, electroblotted, and probed with the GT-ENG-1 CBD antisera at 1:1000 dilution.

RESULTS

Cloning of two EGase cDNA's from G. tabacum: Primers corresponding to *G. rostochiensis* EGase cDNA's (Smant et al., 1998) were used in PCR amplification reactions to isolate corresponding EGase genes in cDNA from J2 of *G. tabacum*. A 3' RACE product of approximately 1.5 kb was amplified using *G. rostochiensis* forward primer CELSIG1.2 (Fig. 1). A single 5' RACE product of 300 bp was amplified using *G. rostochiensis* primer SVP39-INT1 and the nested primer GSVUP (Fig. 1). A database search of the DNA sequence corresponding to the 1.5-kb cDNA product showed strong similarity to family 5 beta-1,4-endoglycanases. The sequence of the 300-bp 5' RACE product was not identical to the 5' end of the 1.5-kb product, suggesting that this fragment corresponded to the 5' end of a second beta-1,4-endoglycanase cDNA. A primer (5' RACEUTR) was designed just upstream of a putative ATG start site on the 300-bp 5' RACE product and used in a 3' RACE reaction to amplify a fragment of approximately 1.3 kb (Fig. 1). Sequencing indicated that this was the full-length cDNA sequence of a second beta-1,4-endoglucanase gene.

The large cDNA clone was 1,540 bp with an open reading frame of 1,413 bp, which encoded a putative precursor protein of 49,710 Da. The first 17 amino acid residues corresponded to a partial signal peptide likely missing several 5' amino acids, because the open reading frame obtained did not contain the ATG start codon. The beta-1,4-endoglucanase catalytic domain spanned amino acids 18 to 324 and was followed by a glycine-serine rich linker domain from residues 325 to 375. Finally, a 95 amino acid carboxy terminal extension showed similarity to type II CBD's (Linder and Teeri, 1997; Fig. 1). The 1,540-bp *G. tabacum* endoglucanase cDNA was designated *GT-eng-1* (Genbank Accession #AF 182392).

The smaller cDNA clone was 1,338 bp with an open reading frame of 1,188 bp, which encoded a putative precursor protein of 42,500 Da. The first 25 amino acid residues corresponded to a putative full-length

signal peptide sequence. The catalytic domain spanned amino acid residues 26 to 330, and a linker domain extended from amino acid residues 331 to 395. This linker harbored a (PAKKPPAKSSKAA)₃ repeat. The smaller cDNA did not contain a predicted CBD (Fig. 1). The 1,338-bp *G. tabacum* endoglucanase cDNA was designated *GT-eng-2* (Genbank Accession #AF182393). *GT-eng-1* and *GT-eng-2* cDNA clones were 92% identical in the first 1,050 nucleotides of their 5' ends.

G. tabacum EGase activity: Degradation of CMC confirmed the presence of cellulolytic activity in *G. tabacum* juveniles. In partially denaturing PAGE separations of total protein from *G. tabacum* J2, a very strong zone of EGase activity (Fig. 2A, lane 1) matched protein at the predicted size for GT-ENG-1 (50 kDa) and a very weak zone of activity detectable to the eye was observed at the predicted protein size for GT-ENG-2 (43 kDa) (Fig. 2A, lane 1, not visible). Antisera raised against recombinant ENG-1 or ENG-2 of *G. rostochiensis* (Smant et al., 1998) were initially tested for cross-reactivity with *G. tabacum* EGases on Western blots. Both *G. rostochiensis* antisera recognized both proteins of the predicted sizes for GT-ENG-1 and GT-ENG-2. A Western blot corresponding to the CMC gel probed with *G. rostochiensis* ENG-1 (GR-ENG-1) antisera confirmed that the proteins exhibiting CMC activity were the same size as GT-ENG-1 and GT-ENG-2 (Fig. 2B, lane 1). *Heterodera glycines* total protein was run as a positive control in the CMC gel overlay assay (Fig. 2A, lane 2). A Western blot probed with HG-ENG-1 antisera showed that the two zones of activity corresponded to the HG-ENG-1 and HG-ENG-2 proteins (Fig. 2B, lane 2). The strong 30-kD band observed in Figure 2B (lane 1) is the result of a cross-reacting protein with the secondary rabbit anti-mouse IgG or goat anti-rabbit IgG alkaline-phosphatase conjugated sera.

GT-ENG-1 and GT-ENG-2 were overexpressed in *E. coli* and purified over nickel affinity columns. The recombinant EGases were approximately 53 kDa (GT-ENG-1) and 46kDa (GT-ENG-2). Both the recombi-

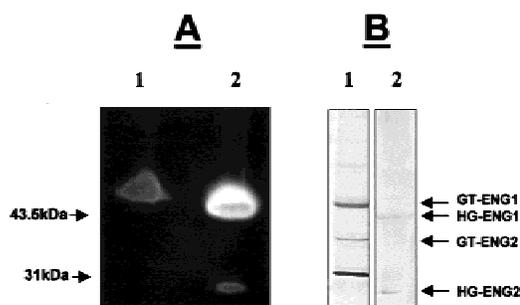


FIG. 2. A) Carboxymethylcellulase activity in a partially denaturing PAGE gel of total proteins separated from second-stage juveniles of *Globodera tabacum* (lane 1) and *Heterodera glycines* (lane 2). B) Corresponding Western blots probed with *G. rostochiensis* ENG-1 antisera (lane 1) and *H. glycines* ENG-1 antisera (lane 2) at 1:5,000 dilution. In Lane 1, antisera raised to *G. rostochiensis* full-length ENG-1 (Smant et al., 1998) bound to both *G. tabacum* ENG-1 and ENG-2, and anti-mouse IgG second antibody bound nonspecifically to a *G. tabacum* protein of about 30kD.

nant GT-ENG-1 and GT-ENG-2 proteins exhibited cellulolytic activity in CMC plate assays (Fig. 3). No zone of activity was observed in the buffer control.

Developmental expression of EGase transcripts: *GT-eng-1* and *GT-eng-2* transcripts were localized during nematode development using in situ hybridization (De Boer et al., 1998). The cellulose binding domain (CBD) of *GT-eng-1* (not present in *GT-eng-2*) was used to construct a riboprobe specific to *GT-eng-1*. Hybridization of sliced nematodes with the antisense *GT-eng-1* CBD riboprobe demonstrated that EGase transcripts accumulated specifically within cells consistent in size and location with the subventral esophageal gland cells of the nematode (Fig. 4). The *GT-eng-1* CBD antisense riboprobe demon-

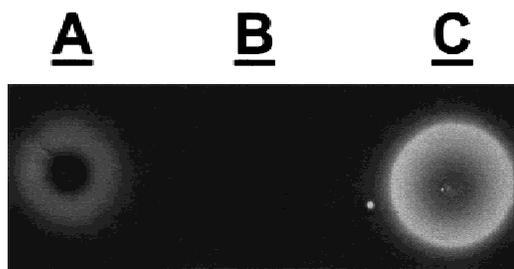


FIG. 3. Degradation of carboxymethylcellulose (clear zones) by affinity-purified recombinant *Globodera tabacum* endoglucanases. A) GT-ENG-1. B) buffer control. C) GT-ENG-2.

strated the presence of *GT-eng-1* mRNA in juveniles within eggs prior to hatching (Fig. 4, panel 1A), in preparasitic J2 (Fig. 4, panel 2A), in migratory parasitic J2 (Fig. 4, panel 3A), in late J4 males prior to emerging from the cuticle (Fig. 4, panel 4A), and in adult males (Fig. 4, panel 5A). *GT-eng-1* transcripts were not detected in J3 males and females in J4 or adult females (data not shown). Sense RNA probes did not hybridize to mRNA in the gland cells of any *G. tabacum* life stage (data not shown). The high percentage nucleotide identity between *GT-eng-1* and *GT-eng-2* did not allow for the construction of a riboprobe specific to *GT-eng-2*. However, an antisense riboprobe constructed to the 5' end of *GT-eng-2* hybridized to the SvG of the same life stages as did the *GT-eng-1* CBD antisense probe, and was not detected in J3 males or females or in J4 and adult females. The results confirm the mRNA accumulation pattern for *GT-eng-1* throughout the nematode life cycle and indicate that neither *GT-eng-1* nor *GT-eng-2* transcripts accumulate in J3 males or females, or in J4 and adult females.

Developmental expression of EGase protein: Immunolocalization studies of *G. tabacum* with GR-ENG-1 antiserum (which binds to GT-ENG-1 and GT-ENG-2) revealed that the pattern of EGase protein accumulation paralleled the observed *GT-eng-1* transcript accumulation. Whenever EGase protein was detected, it was observed in secretory granules throughout the subventral gland cell lobes and along the gland extensions terminating in the ampulla (Fig. 4, panel B). EGase protein was present in juveniles within eggs prior to hatching (Fig. 4, panel 1B), in preparasitic J2 (Fig. 4, panel 2B), and in the migratory parasitic juveniles (Fig. 4, panel 3B). In late sedentary J2, EGase protein levels had either declined significantly or were not detected. EGase protein was not detected in J3 males or females or in J4 females and adult females (data not shown). However, EGases were detectable again in the late J4 males prior to molting (Fig. 4, panel 4B) and in adult male nematodes (Fig. 4, panel 5B).

Efforts were also made to raise polyclonal

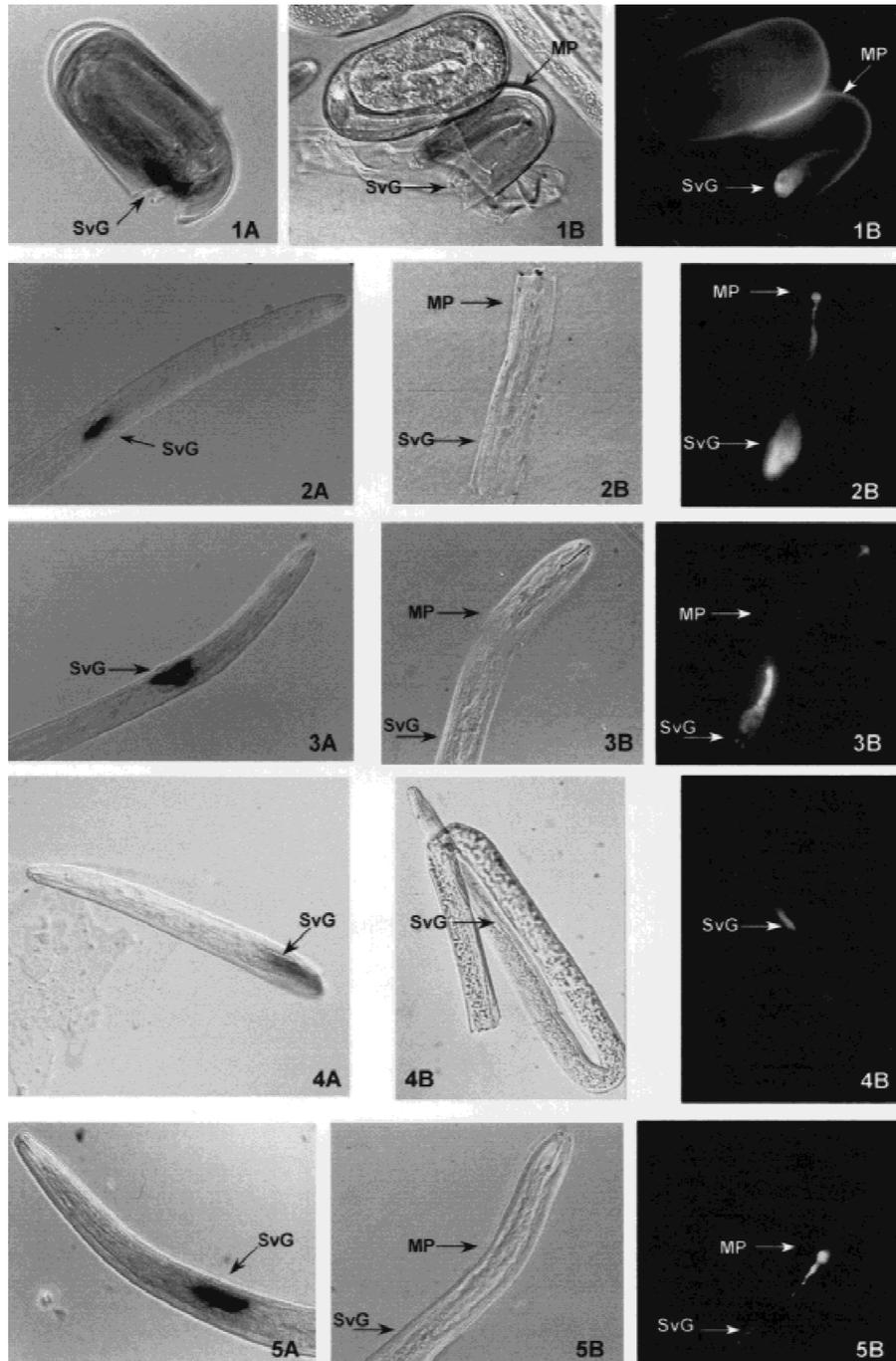


FIG. 4. In situ localization of *Globodera tabacum* GT-eng-1 endoglucanase gene transcripts and the corresponding GT-ENG-1 endoglucanase protein within the nematode during development. Panels labeled "A" depict specimens hybridized with the GT-eng-1 cellulose binding domain antisense probe. Panels labeled "B" represent differential interference contrast (center) and immunofluorescence (FITC secondary antibody) (right) micrographs of the same specimens probed with GR-ENG-1 antisera. 1) second-stage juvenile within eggshell; 2) hatched, preparasitic second-stage juvenile; 3) parasitic second-stage juvenile; 4) fourth-stage male; 5) adult male. EGase transcripts and proteins were not detected in J3 males and females, J4 females, or adult female life stages. Arrows indicate the positions of the subventral esophageal gland cells (SvG) and the metacorporeal pump chamber (MP).

antibodies specific to the GT-ENG-1 CBD (CBD antisera) to better differentiate the expression patterns of each EGase. The GT-ENG-1 CBD antisera bound specifically to GT-ENG-1 on a Western blot of total protein from parasitic *G. tabacum* J2 (Fig. 5). The full-length recombinant GT-ENG-2 protein did not cross-react with the GT-ENG-1 CBD antisera (data not shown).

The CBD antisera was used to immunolocalize GT-ENG-1 protein in parasitic J2 without success. To confirm that GT-ENG-1 protein is secreted, *G. tabacum*-concentrated stylet secretions were subjected to Western blot analysis. The CBD antisera detected a strong band corresponding in size to GT-ENG-1 in stylet secretions from *G. tabacum* J2 induced with DMT (Fig. 6A, lane 2). No bands were detected in similar preparations from nematodes that were not treated with DMT (Fig. 6A, lane 1). A western blot probed with the GR-ENG-1 antisera also detected a protein consistent in size with GT-ENG-2 in stylet secretions. (Fig. 6B, lane 2).

Due to its specificity for GT-ENG-1, the CBD antiserum was used to confirm the developmental expression pattern of GT-ENG-1 on a Western blot of total protein isolated from different *G. tabacum* life stages (Fig. 7). The CBD antisera detected GT-ENG-1 in J2 within eggs (lane 3), in prepara-

sitic J2 (lane 4), in parasitic J2 at 3 days post-inoculation (lane 5), and in adult male nematodes (lane 8). GT-ENG-1 protein was not detected by the CBD antisera in any other developmental stage of *G. tabacum* (lanes 6,7,9, and 10). To account for differences in total protein content among life stages, a separate gel was overloaded with J3 and female total protein preparations. The results were the same as that shown in Figure 7.

DISCUSSION

The tobacco cyst nematode, *G. tabacum*, produces beta-1,4-endoglucanases in the subventral esophageal gland cells, which we hypothesize may function in the hydrolysis of plant cell walls during penetration and intracellular migration of tobacco root tissue. Our results show that the tobacco cyst nematode has at least two beta-1,4-endoglucanase (*eng*) genes, of which we have isolated and sequenced the corresponding cDNA clones, *GT-eng-1* and *GT-eng-2*. *GT-eng-1* and *GT-eng-2* have 96% nucleotide identity and 96% amino acid identity with *eng-1* and *eng-2*, respectively, of the related *G. rostochiensis* (Smant et al., 1998). The *G. tabacum* EGases have approximately 70% amino acid identity with EGases from the soybean cyst nematode, *Heterodera glycines*.

The differences in the predicted protein structure between the two *G. tabacum* EGases suggest that these enzymes may have different substrate specificities. Each nematode EGase contained a signal peptide sequence, a catalytic domain, and a linker domain. The larger of the two EGase gene products (*GT-ENG-1*) contained an additional cellulose-binding domain (CBD) with similarity to type II cellulose-binding domains of bacteria (Linder and Teeri, 1997). Most microbial cellulases contain cellulose-binding domains that confer to the enzyme the ability to bind and degrade crystalline cellulose (Klyosov, 1990). As with potato cyst and soybean cyst nematode EGases, the cellulolytic catalytic domains of the *G. tabacum* EGases had approximately 40% sequence similarity with EGases of soil-dwelling bacteria of the

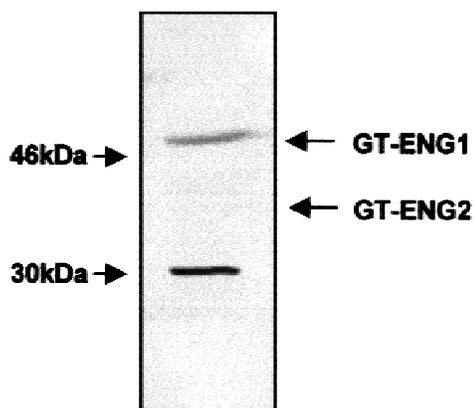


FIG. 5. Antiserum raised to *Globodera tabacum* ENG-1 cellulose binding domain (CBD) binds specifically to GT-ENG-1 on a Western blot of total proteins from second-stage juveniles of *G. tabacum*. Anti-rabbit IgG second antibody bound nonspecifically to a *G. tabacum* protein of about 30kD.

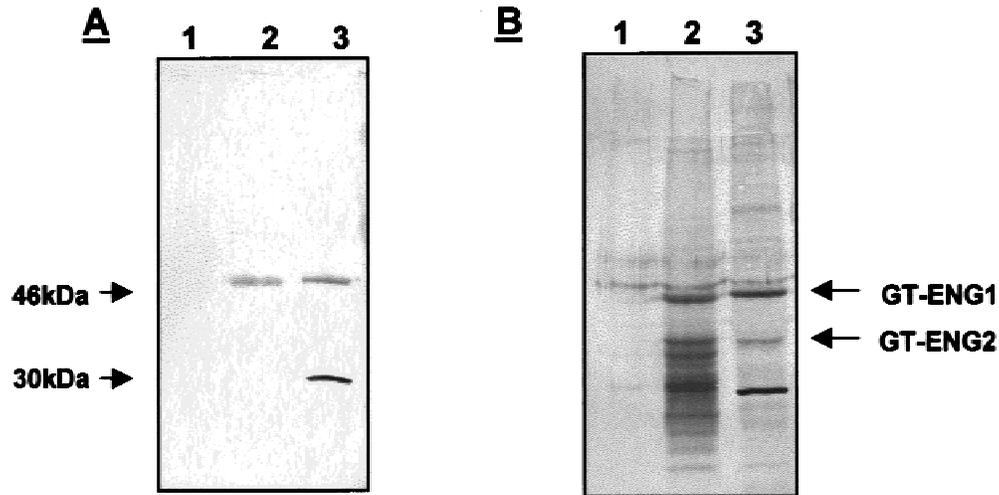


FIG. 6. Western blot of concentrated stylet secretions induced from second-stage juveniles (J2) of *Globodera tabacum* by incubation in 5-methoxy-DMT oxalate. In blots A and B, lanes 1 and 2 contain concentrated, solubilized protein from suspensions of preparasitic J2 that were incubated in water (negative control) and in water plus DMT, respectively. Lane 3 contains total protein from homogenized *G. tabacum* J2. A) Antiserum raised to the cellulose binding domain of GT-ENG-1 binds specifically to GT-ENG-1 in stylet secretions (lane 2); B) Antiserum raised to full-length *G. rostochiensis* ENG-1 (Smant et al., 1998) binds to GT-ENG-1 and GT-ENG-2 in stylet secretions (lane 2). Nonspecific binding of anti-mouse or anti-rabbit IgG second antibody to a 30-kD *G. tabacum* protein (lane 3) is absent in stylet secretions.

genera *Erwinia*, *Clostridium*, and *Bacillus*. These findings have led researchers to speculate that nematode EGases may have been acquired via horizontal gene transfer with bacteria (Davis et al., 2000; Smant et al., 1998; Rosso et al., 1999; Yan et al., 1998).

The ability to degrade carboxymethylcellulose confirmed the enzymatic activity of the predicted *G. tabacum* EGases in nematode homogenates and as recombinant EGases expressed in *E. coli*. In partially denaturing PAGE separations of total protein from *G. tabacum* J2, EGase activity corresponded to a large zone of activity at the predicted protein size for GT-ENG-1 (50

kDa). A very faint zone of activity was observed at the predicted protein size for GT-ENG-2 (43 kDa). The mild denaturation with SDS may have eliminated the activity of GT-ENG-2, since both GT-ENG-1 and GT-ENG-2 exhibited cellulolytic activity when they were overexpressed and purified from *E. coli*.

Localization of EGase transcripts and proteins within the SvG of *G. tabacum* confirmed that the EGases are endogenous to the nematode and developmentally regulated. EGases were expressed within the SvG of J2 within eggs prior to hatching, in hatched preparasitic J2, in parasitic J2, and in late J4

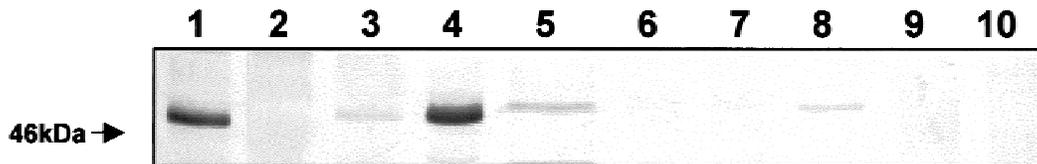


FIG. 7. Western blot of total protein isolated from homogenates of various developmental stages of *Globodera tabacum*. Lane 1) antiserum raised to the full-length ENG-1 endoglucanase of *Globodera rostochiensis* (Smant et al., 1998) binds to ENG-1 from J2 of *G. tabacum* (GT-ENG-1); Lane 2) 46-kD protein molecular weight marker. Total protein from *G. tabacum* developmental stages in Lanes 3–10 was probed with antisera raised to the cellulose binding domain of *G. tabacum* ENG-1; Lane 3) J2 within eggs; Lane 4) preparasitic J2; Lane 5) parasitic J2 at 3 days post-inoculation; Lane 6) parasitic late sedentary J2 at 7 days post-inoculation; Lane 7) J3 males and females; Lane 8) adult males; Lane 9) J4 females; Lane 10) adult females.

male and adult male nematodes. The high percentage of nucleotide and amino acid identity between *GT-eng-1* and *GT-eng-2* made it difficult to differentiate the individual expression patterns of each EGase. The *G. rostochiensis* antiserum made to the full-length recombinant GR-ENG-1 cross-reacted with both *G. tabacum* EGases and was used to monitor both GT-ENG-1 and GT-ENG-2 protein expression during development. The GR-ENG-1 antisera localized EGase production within the subventral esophageal gland cells of *G. tabacum* and confirmed transcription data observed in developmental stages. The GT-ENG-1 CBD antisera did not recognize GT-ENG-1 protein in immunolocalization studies of nematodes even after extensive alterations in the fixation and permeabilization procedures, suggesting that the CBD antiserum was not able to recognize the native form of the protein. The accumulation pattern of GT-ENG-1 as monitored on Westerns probed with the CBD antisera, specifically, confirmed the mRNA in situ and GR-ENG-1 antisera binding observed in developmental stages.

Localization of EGase proteins in the subventral glands of *G. rostochiensis* is confined to preparasitic and parasitic juveniles (Smant et al., 1998). This study of *G. tabacum* provides a more detailed analysis of the developmental regulation of EGases in a *Globodera* species. The results reported here support an immunoblot analysis of protein extracts from *G. rostochiensis* life stages that showed that the MGR48 antigen (an EGase) was present in eggs, preparasitic J2, parasitic J2, and males (Smant et al., 1997). In contrast to the *Globodera* species, *H. glycines* EGase transcripts could be detected in J3 nematodes, but the number of individuals with staining was greatly reduced (De Boer et al., 1999). It is not clear if the *H. glycines* EGases serve a function in the J3 or if they are just residual products (De Boer et al., 1999).

In *G. tabacum*, neither *GT-eng-1* nor *GT-eng-2* EGase transcripts or protein were detected in J3 nematodes. Likewise, *G. rostochiensis* EGases were not detected in J3 total

protein (Smant et al., 1997) preparations, which suggests a potentially tighter regulation of EGase expression in *Globodera* than in *Heterodera* species. The fact that expression was restricted to the mobile stages of the nematode and that expression resumed in the motile male nematodes suggests a potential role for extracellular EGases in penetration, intracellular migration during parasitism of tobacco roots, and emigration of males. Detection of GT-ENG-1 and GT-ENG-2 in secretions from the stylet of *G. tabacum* J2 supports this hypothesis.

Examination of EGase secretion in planta by *G. tabacum* should provide a better understanding of the role of each enzyme during parasitism. Although it is likely that plant enzymes play a major role in the cell wall dissolution that occurs in developing syncytia (Jones and Dropkin, 1975), the possibility exists that nematode EGases may also be secreted into the initial syncytial cells by parasitic J2. Based on the pattern of nematode EGase mRNA and protein expression patterns presented here, however, it appears that these enzymes are not required during the later sedentary stages of the nematode life cycle, at which time the syncytium continues to incorporate additional cells via cell wall dissolution. The evidence suggests that *G. tabacum* provides a model for studying *Globodera* spp. EGases that circumvents U.S. regulatory concerns with *G. rostochiensis* and affords a more tractable plant system (tobacco) than the soybean host of *H. glycines*.

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