MPMI Vol. 12, No. 1, 1999, pp. 64–67. Publication no. M-1998-1109-02N. © 1999 The American Phytopathological Society

Research Note

In Planta Localization of a β-1,4-Endoglucanase Secreted by *Heterodera glycines*

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Accepted 30 September 1998.

Polyclonal sera specific to β-1,4-endoglucanases (cellulases) synthesized in the subventral esophageal gland cells of the soybean cyst nematode, *Heterodera glycines,* were used to provide the first identification of a nematode esophageal gland protein that is secreted into host plant tissue. Sera generated to proteins encoded by *Hg-eng-1* and *Hg-eng-2* (endoglucanases) did not cross-react with soybean root proteins on Western blots (immunoblots) or in immunofluorescence microscopy of noninoculated (control) soybean root sections. In cross sections of soybean roots at 24 h after inoculation of roots with second-stage juveniles of *H. glycines,* HG-ENG-1 was localized within the nematode's subventral gland cells and was not detected in root tissue. HG-ENG-2 was localized within the subventral gland cells and was secreted from the juvenile's stylet into root cortical tissue at 24 h after inoculation of roots with second-stage juveniles of *H. glycines*. HG-ENG-2 was localized along the juvenile's migratory path through the root cortex.

Additional keywords: antibodies, pathogenesis, plant-parasitic nematodes, secretions.

Stylet secretions of plant-parasitic nematodes that originate in the esophageal gland cells are believed to play a major role in pathogenesis and modification of plant cells for feeding (Hussey 1989a; Sijmons et al. 1994). Although changes in the contents, morphology, and activity of the esophageal glands during parasitism of plant roots by nematodes have been well documented (Atkinson and Harris 1989; Bird 1983; Davis et al. 1994; Endo 1987, 1993; Hussey and Mims 1990; Wyss and Zunke 1992; Wyss et al. 1992), the secretion of a nematode esophageal gland protein into host plant tissue has not been clearly demonstrated. Monoclonal antibodies (MAbs) that bind to esophageal gland secretory proteins (Atkinson et al. 1988; Davis et al. 1992; de Boer et al. 1996; Goverse et al. 1994; Hussey 1989b) have not detected nematode esophageal gland proteins that may be secreted into plant tissue, perhaps due to modifications of the MAb-binding epitope of the antigens when they are secreted into host plant tissue. Polyclonal antiserum that recognizes multiple epitopes on a purified nematode antigen provides a better probe than a MAb to identify nematode gland secretions within plant tissue.

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Recently, two β-1,4-endoglucanase (cellulase) cDNAs were identified that are expressed specifically within the subventral esophageal gland cells of the soybean cyst nematode (SCN), *Heterodera glycines* (Smant et al. 1998). A MAb (MGR 48) that bound to a single 49-kDa protein from the subventral glands of *H. glycines* was used to immunoaffinity-purify the protein, and two cDNA products were obtained by reverse transcription-polymerase chain reaction (RT-PCR) with *H. glycines* mRNA in combination with primers derived from the N-terminal amino acid sequence of the purified 49-kDa protein (Smant et al. 1998). *Hg-eng-1* (GenBank accession no. AF006052) encodes an SCN endoglucanase with a catalytic domain that is connected by a peptide linker to a cellulose-binding domain (CBD). *Hg-eng-2* (GenBank accession no. AF006053) encodes an SCN endoglucanase that only contains the catalytic domain. Polyclonal sera generated to HG-ENG-1 and HG-ENG-2 were used to probe sections of soybean roots at 24 h after inoculation with second-stage juveniles (J2) of SCN to demonstrate the secretion of an SCN esophageal gland cell protein into plant tissue, and to begin to elucidate potential roles of the two endoglucanases.

The *Hg-eng-1* and *Hg-eng-2* cDNAs (minus secretion signal peptide sequence) were cloned into the expression vectors pET-28c (at the *Eco*RI site) and pET-28a (at the *Bam*HI and *Xho*I sites), respectively (Novagen, Madison, WI). Overexpressed fusion proteins were affinity-purified under denaturing conditions according to the manufacturer's directions (Novagen), and the purity of the affinity-isolated fusion proteins was confirmed by (10%) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The HG-ENG-1 and HG-ENG-2 bands were excised and used as immunogen. Homogenates of the gel slices (containing 50 to 100 μg of protein) were mixed 1:1 with Freund's adjuvant to immunize rabbits intradermally for polyclonal sera production according to established protocols (Harlow and Lane 1988). The titer and specificity of the sera to HG-ENG-1 and HG-

ENG-2 were determined by enzyme-linked immunosorbent assay (ELISA), Western blots (immunoblots), and immunofluorescence labeling of SCN J2 (Goverse et al. 1994; Hussey et al. 1990).

J2 of SCN were hatched from eggs (Goverse et al. 1994) and used to inoculate soybean cv. Lee 68 root tips (Davis et al. 1989). At 24 h after inoculation, SCN-infected root tips were excised, fixed in 1% paraformaldehyde in dH_2O at room temperature for 6 h, rinsed thoroughly in PBS (137 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8.1 mM Na_2HPO_4, pH 7.4), and placed in 10% dimethylsulfoxide in PBS at room temperature for 30 min for cryoprotection of root tissue. Treated SCN-infected root segments were rinsed thoroughly with dH_2O and blotted to near dryness, positioned for cross-sectioning in liquid HistoPrep frozen embedding medium (Fisher, Pittsburgh, PA) within BEEM capsules (Ted Pella, Inc., Redding, CA), flash-frozen in liquid nitrogen, and stored at –80°C prior to sectioning.

Serial sections of SCN-infected roots (18 μm thick) were cut at –20°C in a Damon/IEC Minotome microtome-cryostat (IEC, Needham Heights, MA), and sections were placed on glass microscope slides that had been coated with 0.01% poly-L-lysine in dH_2O. Primary and secondary antibody solutions were prepared as described previously (Goverse et al. 1994), and sections were rinsed twice with PBS between all treatments. Sections were treated as follows: 30 min in 10% goat serum to block nonspecific antibody-binding sites, 1 h in a 1:250 dilution of polyclonal serum that was generated to either HG-ENG-1 or HG-ENG-2, and 1 h in the dark in a 1:1,000 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO). Uninoculated soybean roots were sectioned and treated as described above to serve as controls. All sections were prepared for observations on an epifluorescence microscope (Zeiss, Oberkochen, Germany) as described previously (Goverse et al. 1994).

The HG-ENG-2 serum bound specifically to an individual protein band of 34 kDa on Western blots of total SCN J2 homogenate (Fig. 1). The HG-ENG-1 serum bound predominantly to a 49-kDa protein band from SCN J2 homogenate, and minimally to the 34-kDa band. In a previous report, cellulase activity was detected in both the 49-kDa and 34-kDa proteins from SCN, and the polyclonal sera generated to HG-ENG-1 and HG-ENG-2 bound specifically within SCN J2 subventral gland cells (Smant et al. 1998). The binding epitope for MGR48 was present in HG-ENG-1 (49 kDa), but it appeared to be absent or unrecognized in HG-ENG-2 (34 kDa) from SCN J2 homogenate. Polyclonal sera to HG-ENG-1 and HG-ENG-2 did not bind to soybean root proteins on Western blots (Fig. 1) or within uninoculated control soybean root sections (Fig. 2A).

The HG-ENG-2 serum bound within SCN J2 subventral gland cells and outside the nematode within soybean root tissue at 24 h after inoculation (Fig. 2B). HG-ENG-2 was usually localized just outside the J2 head, and the occasional binding of the serum along the nematode surface may reflect the deposition of HG-ENG-2 on the cuticle as the J2 migrated forward through the secreted endoglucanase. Binding of the HG-ENG-2 serum within SCN-infected root tissue also was associated with damage to root cortical cell walls along the migratory path of the invading J2 (Fig. 2D). No binding of sera was observed in cell walls that had been mechanically damaged. The HG-ENG-1 serum bound only within the subventral gland cells of J2, and was not detected outside the J2 within root tissue, at 24 h after inoculation of soybean roots with SCN J2 (Fig. 2F).

The specificity of the polyclonal sera unambiguously confirmed the secretion of HG-ENG-2 into soybean root tissue. It has also been demonstrated that the subventral gland antigen recognized by MGR 48 can be secreted from the J2 stylet in in vitro assays (Smant et al. 1997). The observation that HG-ENG-2 was secreted from SCN J2 into root tissue at 24 h after inoculation was unexpected since it was hypothesized that the CBD of HG-ENG-1 would facilitate the nematode's degradation of the crystalline cellulose within plant cell walls during intracellular migration (Smant et al. 1998). A combination of stylet thrusts (Wyss and Zunke 1992) and secreted HG-ENG-2 may be sufficient to promote SCN migration through root cortical cells. It must be stressed that only one time point (24 h) after inoculation has been analyzed thus far, so the potential contributions of HG-ENG-1, HG-ENG-2, and perhaps plant cellulases, during intracellular migration by SCN remain to be determined. The cyst endoglucanases may also be secreted to digest cellulose during the insertion of the nematode stylet tip through the cell wall of the initial syncytial (feeding) cell in the plant vascular tissue (Wyss and Zunke 1992). Evidence suggests that the sedentary plant-parasitic nematode stylet pierces the cell wall, but not the plasmalemma, of the feeding cell (Williamson and Hussey 1996). Modification of root vascular cells into a syncytium (permanent feeding site) by a cyst nematode involves extensive cell wall dissolution of neighboring plant cells, and it is hypothesized that the cell wall-degrading enzymes involved in syncytium formation are of plant origin (Jones 1981). The MGR 48 (endoglucanase) antigen has been detected in parasitic J2 and males of *G. rosto-*

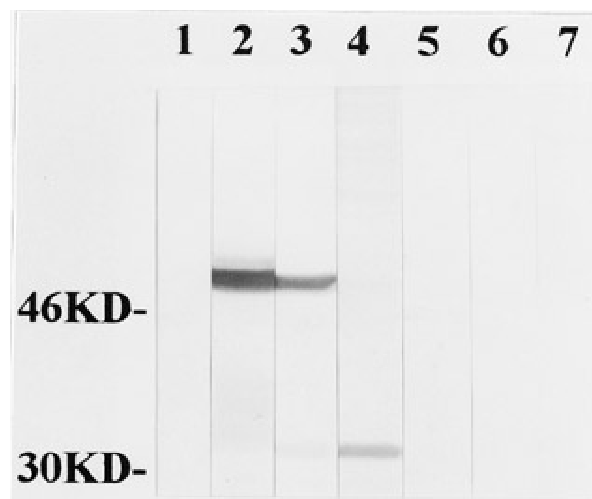
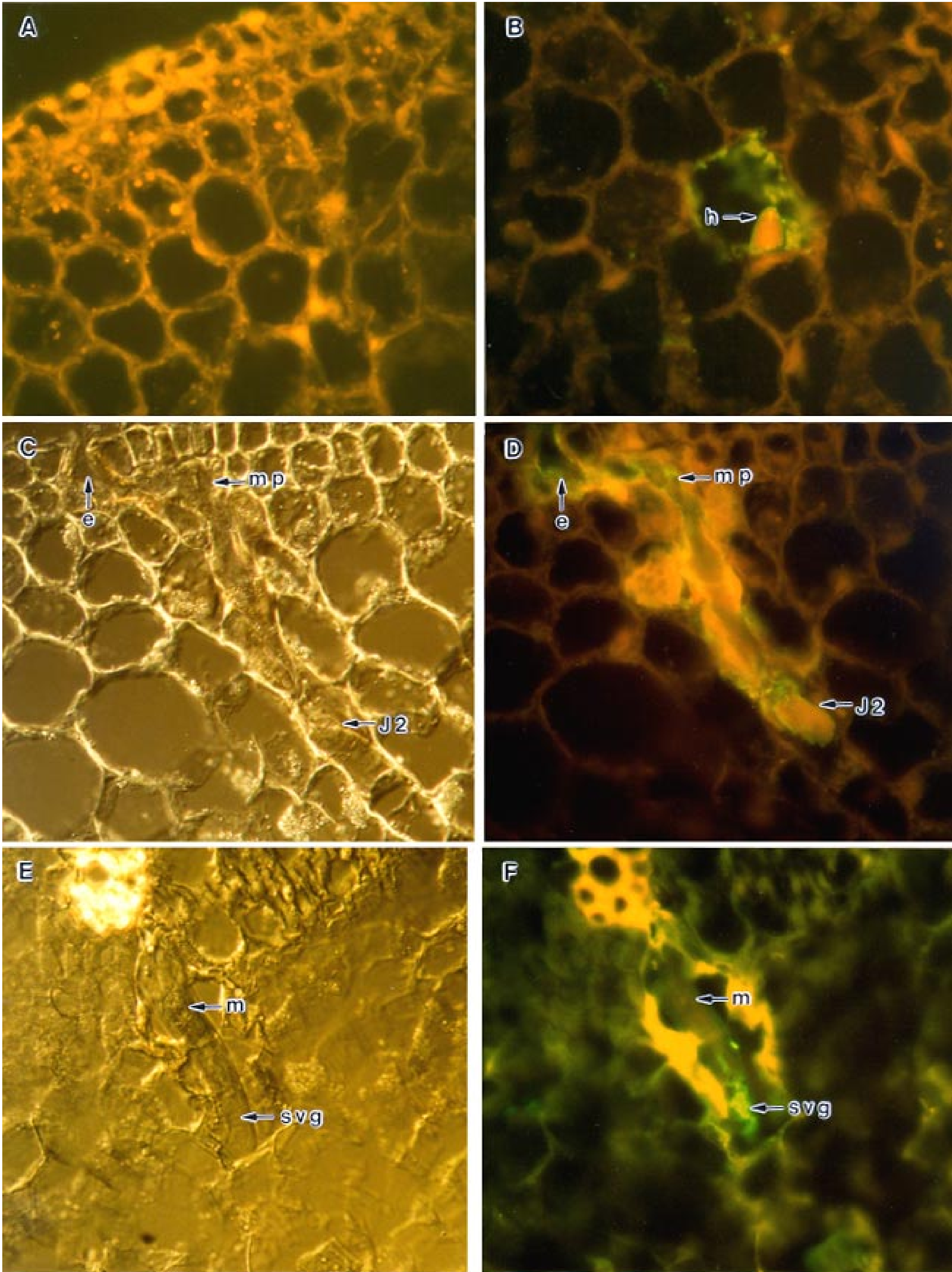


Fig. 1. Western blot (immunoblots) of proteins from homogenates of second-stage juveniles of *Heterodera glycines* (lanes 1–4) and proteins from homogenates of noninoculated soybean roots (lanes 5–7) probed with antibodies to *H. glycines* (HG-ENG-1 and HG-ENG-2) β-1,4-endoglucanases. Lane 1: Pre-immune rabbit serum; lane 2: MGR 48 monoclonal antibody; lane 3: antiserum to HG-ENG-1 endoglucanase; lane 4: antiserum to HG-ENG-2 endoglucanase; lane 5: pre-immune rabbit serum; lane 6: antiserum to HG-ENG-1 endoglucanase; and lane 7: antiserum to HG-ENG-2 endoglucanase.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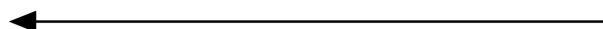

Fig. 2. Indirect immunofluorescence (fluorescein isothiocyanate: FITC) microscopy of soybean root cross sections at 24 h after inoculation of roots with second-stage juveniles (J2) of *Heterodera glycines* and probed with antisera that bind to β-1,4-endoglucanases from *H. glycines*. **A,** No binding of HG-ENG-2 antiserum within an uninoculated control root. **B,** Binding of HG-ENG-2 antiserum (green fluorescence) to the endoglucanase secreted into root tissue around the head (h) of a J2. **C,** Light micrograph showing root cortical tissue disruption along the migratory path (mp) of a J2 from its point of entry at the root epidermis (e). **D,** Same section as **C** showing binding of HG-ENG-2 antiserum (green fluorescence) associated with tissue disruption along the migratory path of the J2. **E,** J2 in root cortex with metacorpus (m) and subventral esophageal glands (svg) visible. **F,** Same section as **E** showing binding of HG-ENG-1 antiserum (green fluorescence) within the subventral glands of *H. glycines*.

chiensis, but not in J3, J4, or adult female stages (Smant et al. 1997). Future experiments to monitor and localize the temporal and spatial expression of the endoglucanases during the entire cyst nematode parasitic cycle will elucidate the roles of these enzymes during plant pathogenesis.

ACKNOWLEDGMENTS

This research was supported by the North Carolina Agricultural Research Service, the U.S. Department of Agriculture under NRI grant 95-37302-1918 and FAS/ICD/RSED grant 59-3148-6-003, the Iowa Agriculture and Home Economics Experiment Station (J-18094, Project No. 3381), and the United Soybean Board under grant 400-41-52. In addition, support was provided from the Dutch Potato Board, the Dutch Technology Foundation coordinated by the Life Science Foundation, European Community grants BIO2-CT92-0239, BIO4-CT96-0318, and FAIR1-CT95-0905, and North Atlantic Treaty Organization Award CRG931004.

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