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Identification of a New β-1,4-endoglucanase Gene Expressed in the Esophageal Subventral Gland Cells of *Heterodera glycines*[1]

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Abstract: Secretory proteins encoded by parasitism genes expressed in the esophageal gland cells of plant-parasitic nematodes play key roles in nematode-plant interactions. A fourth β-1,4-endoglucanase full-length cDNA (designated *Hg-eng-4*) was isolated from a *Heterodera glycines* esophageal gland-cell long-distance polymerase chain reaction cDNA library. The cDNA hybridized to genomic DNA of *H. glycines* in Southern blots. The *Hg-eng-4* cDNA contained an open reading frame encoding 352 amino acids, with the first 18 amino acids being a putative secretion signal. Hg-ENG-4 contained a family 5 endoglucanase catalytic domain and a peptide linker of repeat amino acids, but no cellulose binding domain. In-situ hybridization analyses showed that transcripts of *Hg-eng-4* accumulated specifically in the subventral gland cells of pre-parasitic and parasitic second-stage juveniles of *H. glycines.*

Key words: cDNA, cellulase, cyst nematode, esophageal gland cell, *Heterodera glycines,* parasitism gene.

Cloning parasitism genes encoding proteins secreted from the esophageal gland cells and injected through the stylet into plant tissue is essential for understanding the molecular basis of nematode parasitism of plants (Davis et al., 2000). The first parasitism genes encoding esophageal gland cell secretory proteins to be cloned from plant-parasitic nematodes were β-1,4-endoglucanases (cellulases) from *Heterodera glycines* and *Globodera rostochiensis* (Smant et al., 1998; Yan et al., 1998). Cellulase genes also have been cloned from *Meloidogyne incognita* (Rosso et al., 1999), *Globodera tabacum* (Goellner et al., 2000), and *Heterodera schactii* (de Meutter et al., 2001). The cellulases are synthesized in a developmental pattern in the subventral gland cells of *Heterodera glycines* (de Boer et al., 1999) and secreted within the host tissue by infective juveniles during the penetration and intracellular migration phases of the infection process (Wang et al., 1999). Three cellulase genes (*Hg-eng-1, Hg-eng-2,* and *Hg-eng-3*) have been cloned from *H. glycines* (Smant et al., 1998; Yan et al., 2001). *Hg-eng-1* encodes an endoglucanase with a catalytic domain that is connected by a peptide linker to a cellulose binding domain. *Hg-eng-2* and *Hg-eng-3* encode endoglucanases that contain only the catalytic domain. Here we report the cloning of a cDNA that encodes a new endoglucanase that contains a catalytic domain and a peptide linker and is expressed in the subventral gland cells of *H. glycines.*

Materials and Methods

Heterodera glycines Ichinohe was cultured on greenhouse-grown soybean (*Glycine max* (L.) Merr.). Pre-parasitic second-stage juveniles were collected by hatching eggs on 25-μm-pore sieves in deionized water in plastic bowls. Parasitic stages of *H. glycines* were collected 8 days after inoculation by root blending and sieving (de Boer et al., 1999).

Isolation of cDNA clones: A full-length cDNA (clone 6F06) encoding a β-1,4-endoglucanase, designated *Hg-eng-4* (GenBank Accession #AY043224), was identified during random sequencing of clones from a long-distance (LD) polymerase chain reaction (PCR) cDNA library (Clontech Laboratories, Inc., Palo Alto, CA). This library was derived from mRNA from esophageal gland cell cytoplasm of *H. glycines* parasitic stages (Gao et al., 2001). Sequence of full-length *Hg-eng-4* cDNA in pGEM-T Easy vector was obtained using T7 and SP6 primers in sequencing reactions.

Southern hybridization: Genomic DNA of *H. glycines* (10 μg) was digested with *Bam*HI, fractionated in 1% agarose gels, and then transferred onto Hybond-N Nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) (Ray et al., 1994). A hybridization probe specific for *Hg-eng-4* was prepared by using forward primer 6F06F (5′-CAACACAAGAAAAAATCCACCGGT-3′) and reverse primer 6F06R (5′-TGATTTGCTGATCATCCGGAC-3′) to amplify a cDNA fragment (146-bp) corresponding to the linker domain from the LD PCR library clone 6F06 (Fig. 1). The probe was labeled with ^{32}P-dCTP using the RTS RadPrime DNA Labeling System (GIBCO-BRL, Grand Island, NY). Hybridizations were performed overnight at 68 °C, and membranes were washed with 0.1 × SSC and 0.1% SDS at 68 °C according to standard procedures (Sambrook et al., 1989). Films were exposed overnight at −80 °C prior to developing.

In-situ hybridization: The same 146-bp cDNA fragment used in the Southern hybridization was also used for in-situ hybridization experiments. Synthesis of digoxigenin-labeled sense and antisense cDNA probes (Boehringer Mannheim Corp., Penzberg, Germany) by asymmetric PCR amplification (Wang et al., 2001) was conducted using primers 6F06F and 6F06R.

In-situ hybridization was performed as described by de Boer et al. (1998). Pre-parasitic and mixed parasitic

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   1                                             ccaactaattttctcccaat 20
  21 ctaaaaaagtctctctaattaatactaattagtacaaactgctgttgatttgatcacaga 80
  81 ATGTTGGTTCAACTCGTCCTCCTTGCCATCATTGGCATTTCCTTTGTCGGTGCTGCCGCG 140
   1 M  L  V  Q  L  V  L  L  A  I  I  G  I  S  F  V  G  A  A  A   20

 141 CCGCCGTACGGCCAATTGTCCGTCTCCGGCACCAAATTGGTTGGCTCAAACGGCAAACCG 200
  21 P  P  Y  G  Q  L  S  V  S  G  T  K  L  V  G  S  N  G  K  P   40

 201 GTGCAGCTGATCGGCAATTCGTTGTTCTGGCACCAGTGGTACCCACAATTTTGGAATACT 260
  41 V  Q  L  I  G  N  S  L  F  W  H  Q  W  Y  P  Q  F  W  N  T   60

 261 GAAACAGTGAAGGCACTCAAATGCAATTGGAATTCCAATGTCGTGCGCACCGCAATGGGC 320
  61 E  T  V  K  A  L  K  C  N  W  N  S  N  V  V  R  T  A  M  G   80

 321 GTGGAACAGGGCGGCTATCTGAGTGACGCGAACACCGCCTACCGACTGACGGCAGCTGTG 380
  81 V  E  Q  G  G  Y  L  S  D  A  N  T  A  Y  R  L  T  A  A  V   100

 381 ATTGAGGCGGCCATTGCACAGGGCATTTACGTGATCGTCGATTGGCACGCGCATGAGGCG 440
 101 I  E  A  A  I  A  Q  G  I  Y  V  I  V  D  W  H  A  H  E  A   120

 441 AACGCGGACAAAGCGATTGAATTCTTCACCAAAGTTGCGAAAGCGTACGGCTCCAACCCT 500
 121 N  A  D  K  A  I  E  F  F  T  K  V  A  K  A  Y  G  S  N  P   140

 501 CACTTGCTTTACGAAACGTTTAACGAGCCGTTGGACGTGTCTTGGAACGATGTGCTTGTC 560
 141 H  L  L  Y  E  T  F  N  E  P  L  D  V  S  W  N  D  V  L  V   160

 561 CCGTACCATAAAAAGGTTATTTCTGCAATTCGTGCCATCGACAAAAAGAATGTGATCATT 620
 161 P  Y  H  K  K  V  I  S  A  I  R  A  I  D  K  K  N  V  I  I   180

 621 CTCGGCACTCCCAAATGGTCTCAAGATGTTGACGTGGCGGCCCAAAATCCGATCAAAGGA 680
 181 L  G  T  P  K  W  S  Q  D  V  D  V  A  A  Q  N  P  I  K  G   200

 681 TTCGGTAATTTGATGTACACTCTCCACTTCTATGCGTCCAGTCACTTTGTTGATGGACTT 740
 201 F  G  N  L  M  Y  T  L  H  F  Y  A  S  S  H  F  V  D  G  L   220

 741 GGAAATAAGCTTAAGACCGCCGTAAACAAGGGTCTTCCGGTGTTCGTCACTGAGTACGGT 800
 221 G  N  K  L  K  T  A  V  N  K  G  L  P  V  F  V  T  E  Y  G   240

 801 ACATGCGAAGCGTCTGGCAATGGTAATCTGAATACCAATTCAATGTCAAGCTGGTGGAGC 860
 241 T  C  E  A  S  G  N  G  N  L  N  T  N  S  M  S  S  W  W  S   260

 861 CTGCTGGACCAACTGCAAATTTCGTACGTCAATTGGTCAATCACTGACAAAAGCGAAGCT 920
 261 L  L  D  Q  L  Q  I  S  Y  V  N  W  S  I  T  D  K  S  E  A   280

 921 TGTGCAGCGCTCACTGGCGGAACATCGGCTGCCAATGTTGGCACTTCCTCCCGCTGGACG 980
 281 C  A  A  L  T  G  G  T  S  A  A  N  V  G  T  S  S  R  W  T   300
                                      6F06F
 981 CAGTCTGGCAATATGGTAGCTTCGCAACACAAGAAAAAATCCACCGGTGTGAACTGCAGC 1040
 301 Q  S  G  N  M  V  A  S  Q  H  K  K  K  S  T  G  V  N  C  S   320

1041 GGTGGTGGTGGCGCTGCTGCTAAGCCAGCTGCTAAGCCCGCCGCTAAGCCAGCTGCTAAA 1100
 321 G  G  G  G  A  A  A  K  P  A  A  K  P  A  A  K  P  A  A  K   340
                                        6F06R
1101 TCGAAGGGAAAGTCTTCCAAAGCCAAGAAGTCCGGATGAtcagcaaatcacaataaacat 1160
 341 S  K  G  K  S  S  K  A  K  K  S  G  *                        352

1161 agaaagtgaattgaagacaatatggtgattcaaaaaacaaataagtgcataatgataatt 1220
1221 ttaagtataattgtaatattcaaaaatattcttaggagtaaatcgggcactgataaagca 1280
1281 tgaactattattaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa          1333
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Fig. 1. Complete nucleotide and deduced amino acid sequences of the *Heterodera glycines Hg-eng-4* cDNA. The predicted 18 amino acid secretion signal sequence is indicated in bold type with the putative protease cleavage dotted underlined. The linker domain is indicated in bold italics. The two putative *N*-linked glycosylation sites (Asn271 and Asn318) are double underlined. The gene-specific primers (6F06F and 6F06R) used for amplification of the fragment used for in-situ hybridization are underlined. The TGA stop codon is marked with an asterisk, and the putative polyadenylation signal sequence (aataaa) is underlined.

TABLE 1. Homology of a β-1,4-endoglucanase (Hg-ENG-4) from *Heterodera glycines* with other endoglucanases from cyst nematodes.

Enzyme[a]	Nematode	Accession no.	Catalytic domain Similarity (%)	Catalytic domain Identity (%)	Total no. amino acids
Hg-ENG-3	*H. glycines*	AF044210	97	96	319
Hg-ENG-2	*H. glycines*	AF006053	97	96	319
Hs-ENG-2	*H. schachtii*	AJ299387	98	97	365
Hg-ENG-1	*H. glycines*	AF006052	90	80	476
Gr-ENG-2	*G. rostochiensis*	AF004712	87	74	392
Gt-ENG-2	*G. tabacum*	AF182393	87	74	395
Gr-ENG-1	*G. rostochiensis*	AF004523	86	74	472
Gt-ENG-1	*G. tabacum*	AF182392	86	73	470

[a] Hg - *Heterodera glycines,* Hs - *H. schachtii,* Gr - *Globodera rostochiensis,* Gt - *G. tabacum.*

stages of *H. glycines,* washed three times with 1× phosphate buffered saline, were fixed in 10% formalin buffered in phosphate (pH 7.4) for 2 days at room temperature. Fixed nematodes were randomly sliced on a glass slide with a razor blade until approximately 90% of the nematodes were cut. Nematode sections were permeabilized with 500 ng/ml proteinase-K (Boehringer Mannheim, Inc., Penzberg, Germany) at room temperature for 1 hour. After pre-hybridization, nematode sections in hybridization buffer were aliquoted to wells of a 96-well MultiScreen plate (Millipore, Bedford, MA). Denatured PCR digoxigenin-labeled DNA probes (1 μl) were added to each well. Hybridization was performed overnight at 55 °C. Stringent wash solutions were changed by aspiration through the well bottoms using the Millipore MultiScreen vacuum manifold (Gao et al., 2001). Hybridization signal within the nematode sections was detected by light microscopy.

Results and Discussion

The full-length *Hg-eng-4* cDNA contained 1,157 nucleotides (excluding the poly (dA) tail) with a putative open reading frame (ORF) of 1,056 bp (Fig. 1). The cDNA contained 80 bp of 5′ UTR and 18 bp of 3′ UTR, which contained a polyadenlylation signal (AATAAA). The ORF encoded a deduced protein of 352 amino acids with a calculated molecular weight of 37,587 and a pI of 9.4, as predicted by the ProtParam tool (Gill and von Hippel, 1989). A putative signal sequence, predicted by SignalP, terminated immediately upstream of a putative protease cleavage site between amino acids Ala18 and Ala19 (Nielsen et al., 1997). PSORT II (Nakai and Horton, 1999) predicted Hg-ENG-4 to be extracellular. There were two putative N-glycosylation sites at amino acids Asn271 and Asn318 as predicted by PROSITE (Hofmann et al., 1999). In high-stringency Southern blot hybridizations with *Bam*HI-digested genomic DNA from *H. glycines, Hg-eng-4* cDNA hybridized to one major band (~5.5 kb) (data not presented).

BLAST searches (Benson et al., 1998) revealed the predicted Hg-ENG-4 amino acid sequence had the highest similarities with β-1,4-endoglucanases Hg-ENG-2 and Hg-ENG-3 (97% similarity and 96% identity in the catalytic domain) from *H. glycines* and Hs-ENG-2 (98% similarity and 97% identity) from *H. schachtii* (Table 1). Hg-ENG-4 contains a predicted 5′ secretion signal peptide, a family 5 endoglucanase catalytic domain, and a peptide linker of repeat amino acids—a structure similar to Hs-ENG-2, Gt-ENG-2, and Gr-ENG-2, in *H. schachtii, G. tabacum,* and *G. rostochiensis,* respectively (de Meutter et al., 2001; Goellner et al., 2000; Smant et al., 1998). Interestingly, the sequence of the peptide linker in Hg-ENG-4 had higher similarity with the linker in Hs-ENG-2 (75%) than with the linker in Hg-ENG-1 (22%). Hg-ENG-4 is the first cellulase cloned from *H. glycines* that contains the catalytic domain and the peptide linker without the cellulose binding domain. As with the other plant-parasitic nematode cellulases (Yan et al., 1998), Hg-ENG-4 had significant similarities with bacterial cellulases (e.g., β-1,4-endoglucanase from *Pseudomonas fluorescens,* GenBank accession no. AFS56132, 230/e^{59}, amino acid sequence identity 51%).

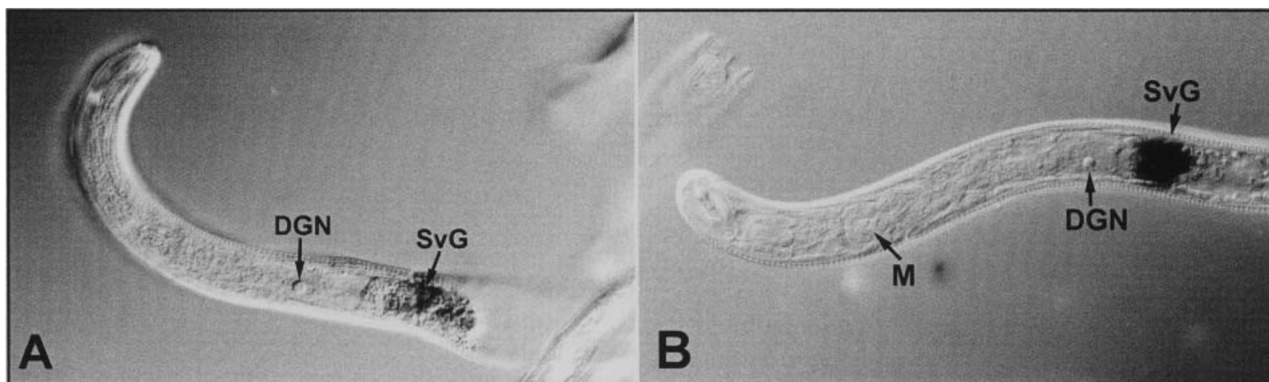

FIG. 2. Hybridization of a digoxygenin-labeled antisense cDNA probe (dark staining) of *Hg-eng-4* to transcripts expressed exclusively within the subventral esophageal gland cells of *Heterodera glycines.* A) Pre-parasitic second-stage juvenile. B) Migratory parasitic second-stage juvenile. SvG) Subventral gland cells. M) Metacorpus. DGN) Dorsal gland nucleus.

In-situ hybridization showed that the digoxigenin-labeled antisense cDNA probes of *Hg-eng-4* specifically hybridized with *Hg-eng-4* transcripts within the subventral esophageal gland cells of *H. glycines* (Fig. 2). The hybridization signal was consistently weak within the subventral gland cells in the pre-parasitic second-stage juveniles (Fig. 2A) and intensified in migratory parasitic second-stage juveniles (Fig. 2B). Transcripts of *Hg-eng-4* were rarely detected within the subventral gland cells in sedentary second-stage juveniles and not detected in third-stage juveniles or later stages (data not shown). The expression profile for *Hg-eng-4* mirrors the degeneration of the subventral gland cells in *H. glycines* during the late stages of parasitism (de Boer et al., 1999). No hybridization with mRNA was observed within sections of *H. glycines* with the control sense cDNA probe of *Hg-eng-4* (data not shown). The developmental expression pattern of cellulases in *H. glycines* indicates they have a role in softening soybean cell walls during penetration and migration through root tissues by infective second-stage juveniles (de Boer et al., 1999; Wang et al., 1999).

β-1,4-endoglucanases likely constitute a family of secreted endogluconases in *H. glycines,* although the number of family members has not been ascertained. Second-stage juveniles of *H. glycines* apparently employ an arsenal of β-1,4-endoglucanases to hydrolize the β-1,4 glycosidic bonds of cellulose in the cell walls during their intracellular migration within soybean roots (Wang et al., 1999). Because it is not clear whether each β-1,4-endoglucanase identified to date is differentially expressed in *H. glycines* during parasitism, the specific role for each enzyme remains unknown.

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