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Cloning of a Putative Pectate Lyase Gene Expressed in the Subventral Esophageal Glands of *Heterodera glycines*[1]

J. M. de Boer,[2] J. P. McDermott,[2] E. L. Davis,[3] R. S. Hussey,[4] H. Popeijus,[5] G. Smant,[5] and T. J. Baum[2]

Abstract: We report the cloning of a *Heterodera glycines* cDNA that has 72% identity at the amino acid level to a pectate lyase from *Globodera rostochiensis*. In situ hybridizations showed that the corresponding gene (*Hg-pel-1*) is expressed in the subventral esophageal gland cells of second-stage juveniles. The deduced amino acid sequence of the *H. glycines* cDNA shows homology to class III pectate lyases of bacterial and fungal origin.

Key words: esophageal gland, molecular nematology, nematode, pectate lyase gene.

Secretions produced in the dorsal and subventral esophageal gland cells of cyst nematodes and root-knot nematodes play an essential role in the interaction of these plant parasites with their host (Hussey, 1989). Presumed functions of these gland secretions include (i) cell wall maceration during the initial migration of the nematodes within the root tissue, (ii) induction and maintenance of feeding cells within the root tissue, and (iii) assistance with food uptake from these feeding cells (Hussey, 1989).

Beta-1,4-endoglucanase (EGase) genes have been identified in five species of endoparasitic nematodes, and all show sequence similarity on the amino acid level to EGases of bacterial origin (De Meutter et al., 1998; Goellner et al., 2000; Rosso et al., 1999; Smant et al., 1998; Yan et al., 2001). EGases hydrolyse the β-1,4-glucan bonds in polysaccharides such as cellulose and xyloglucan, which constitute the major components of the plant primary cell wall. In endoparasitic nematodes, EGase transcripts and translation products are abundantly expressed in the subventral gland cells of second-stage juveniles (J2) and males (De Boer et al., 1996, 1999; Goellner et al., 2000; Rosso et al., 1999). The nematode EGases have been shown to be secreted by J2, both in vitro and while penetrating roots of the host plant (De Meutter et al., 1998; Rosso et al., 1999; Smant et al., 1997; Wang et al., 1999). These observations indicate that endoparasitic nematodes secrete EGases to soften the plant cell walls to facilitate their migration within the root tissue.

Recently, a second category of cell wall degrading enzymes was found to exist in a plant-parasitic nematode. Specifically, a pectate lyase cDNA was identified from J2 of *G. rostochiensis* (Popeijus et al., 2000). Pectate lyases depolymerize the pectic component of the primary plant cell wall and middle lamella by β-elimination of the glycosidic bonds. In *G. rostochiensis,* the pectate lyase gene (*Gr-pel-1*) was found to be transcribed in the subventral esophageal gland cells of J2. Furthermore, heterologous overexpression of *Gr-pel-1* yielded an active enzyme. It was therefore proposed that *G. rostochiensis* secretes a mixture of cellulase and pectinase enzymes to attack the plant cell wall (Popeijus et al., 2000). Here we report the cloning of a similar pectate lyase cDNA from *H. glycines*.

Materials and Methods

cDNA library screening: An oligo(dT) primed cDNA library, containing 1.5×10^{10} pfu/ml, was constructed from J2 of *H. glycines* in the Uni-ZAP XR vector (Stratagene, La Jolla, CA). Phage were plated out on four 15-cm NZC petri dishes at a density of 3.0×10^4 pfu/plate and grown at 37 °C for 6 hours when the plaques were 1.0–2.0 mm in diam. After cooling to 4 °C, the plaques were transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ).

A ^{32}P-labeled probe was prepared from the *Gr-pel-1* cDNA (Popeijus et al., 2000) by PCR, using the gene-specific primers grpelf (5′-CCATCACAGTACAAGC-3′) and pecr1 (5′-GGTTGGTCTGAATTTCGCAT-3′) to generate a 681-bp fragment. Amplification was in a 20-µl reaction containing Ex Taq Buffer (Panvera, Madison, WI); 3.0 µM each of dATP, dGTP, and dTTP, 0.5 µM grpelf primer, 0.5 µM pecr1 primer, 1 ng denatured *Gr-pel-1* template, 0.1 U/µl Ex Taq DNA polymerase (Panvera, Madison, WI); and 0.825 µM alpha-^{32}P-dCTP (Amersham Pharmacia Biotech, Piscataway, NJ). The cycling conditions were 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute, and a final step at 72 °C for 10 minutes. Unincorporated label was removed using a QIAquick Nucleotide Removal Kit (Qiagen Inc, Valencia, CA).

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[2] Post-doctoral Researcher, Research Associate and Associate Professor, respectively, Department of Plant Pathology, Iowa State University, 351 Bessey Hall, Ames, IA 50011.
[3] Associate Professor, Department of Plant Pathology, North Carolina State University, 1515 Gardner Hall, Raleigh, NC 27695.
[4] Research Professor, Department of Plant Pathology, University of Georgia, Athens, GA 30602-7274.
[5] Ph.D. student and Assistant Professor, respectively, Laboratory of Nematology, Graduate School of Experimental Plant Sciences, Department of Plant Science, Wageningen University, Binnenhaven 10, 6708 PD Wageningen, The Netherlands.
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E-mail: tbaum@iastate.edu
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GR-PEL-1        MLFVIISIVFAQIFQVHA-LCTFPSSTKTITVQATMNVASNTDYKYTTFVGGSGILNGAC 59
HG-PEL-1        --FILLVITFVQIGQLNAGICTFPNPSKSVTVQSMMTVSSSTDYKNTLFVGGSGILNGAC 58
                                    ^
GR-PEL-1        DVKNGKMKYLMVLKHGVTIKNAIINTPGLGIYCEGSCVLENIYYKKLCYHATGFGYKSTG 119
HG-PEL-1        DVNNDKLKYLMTLKNGVTIKNAILDTPGLGIYCEGNCVLENIYYKRLCYHATGFGYKSTS 118

GR-PEL-1        TSYTYQVIGGAGQGSPDKYFTQSGRGTTIIKNFCAEGKYGKVWCSCGNCIDQMPRSVQIS 179
HG-PEL-1        TSYTYQVIGGAGQGSPDKYFTQSGKGTTIIKNFCAEGKYGKLWCSCGNCPFQTARTVQIS 178

GR-PEL-1        NTKIQGPGLAIISANSNYGDKISISGLTLYGQGSPNTLTKYICQSYNGLTTMATMQPNAK 239
HG-PEL-1        NTVLKGPGLSVVSLNSNYGDKMSLSGLTLHGQKSASTKTSYICQEYKGLTYMAAMSPQAN 238

GR-PEL-1        FRPTQSGTGTCSYSTSAIKIVN 261
HG-PEL-1        YEPTKSGSGTCAYSASAVKIAS 260
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FIG. 1. Clustal-W alignment of the deduced amino acid sequences of *Hg-pel-1* (AY026357) and *Gr-pel-1* (AAF80746). The predicted signal peptides are underlined. The arrow indicates a consensus N-glycosylation site in HG-PEL-1. Colons indicate conserved substitutions; dots indicate semi-conserved substitutions.

Membranes were prehybridized overnight at 42 °C in hybridization buffer (50% deionized formamide, 5X SSC, 25 mM sodium phosphate buffer pH 6.8, 0.1% SDS, 1X Denhardt's solution, 0.1 mg/ml denatured carrier DNA, and 10% dextran sulfate) and hybridized overnight at 42 °C with *Gr-pel-1* probe in hybridization buffer. The membranes then were washed twice in a solution containing 2X SSC and 0.1% SDS for 30 minutes at 37 °C, followed by two washes in a solution containing 0.1X SSC and 0.1% SDS for 30 minutes at 55 °C. Positive clones were identified by exposing BioMax MR film (Kodak, Rochester, NY) to the membranes. The positive clones were resuspended in 1.0 ml SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, and 0.01% gelatin) with 40 µl chloroform. Phage were then plated out, and subsequent rounds of enriching the clones were carried out similarly as described above.

Sequence analysis: cDNA samples were sequenced at the DNA Sequencing and Synthesis Facility of Iowa State University. Similarity searches in the GenBank database were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using the BLASTP algorithm, version 2.1 (Altschul et al., 1997), using default settings. Clustal-W protein sequence alignment was performed at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/). The program SignalP V2.0 (Nielsen et al., 1997) was used to search for a signal peptide sequence.

In situ hybridization: Digoxigenin-labeled sense and antisense RNA probes were synthesized by run-off transcription from linearized plasmid containing the target cDNA. The RNA probes were ethanol-precipitated and subjected to alkaline hydrolysis at pH 10.2 to produce probe fragments of 150 bp average length. The hydrolyzed probes were cleaned by ethanol precipitation, resuspended in water, and used for in situ hybridization, as described previously (De Boer et al., 1998).

RESULTS AND DISCUSSION

Plaque lift membranes from an *H. glycines* J2 cDNA library were hybridized with a radioactively labeled DNA probe prepared from the *Gr-pel-1* cDNA. The *G. rostochiensis* probe hybridized to eight *H. glycines* cDNAs, which ranged in size from 500 to 1,000 base pairs (bp). DNA sequencing of these cDNA fragments showed that

TABLE 1. Similarities of the deduced protein sequence of *Hg-pel-1* to Class III pectate lyases (Shevchik et al., 1997) of fungal and bacterial origin.

Accession	Protein description	Identities	P-value
CAA73784	endo-pectate lyase I [*Pectobacterium chrysanthemi*]	68/236 (28%)	8e-17
CAA55814	pectate lyase B [*Pectobacterium carotovorum*]	67/222 (30%)	1e-13
S68364	pectate lyase C [*Fusarium solani* f. sp. *pisi*]	58/187 (31%)	7e-13
AAA57140	pectate lyase 3 [*Pectobacterium carotovorum*]	59/191 (30%)	3e-12
AAC64368	pectate lyase 1 [*Fusarium oxysporum* f. sp. *lycopersici*]	69/230 (30%)	5e-11
AAA87383	pectate lyase B [*Fusarium solani* f. sp. *pisi*]	50/158 (31%)	1e-10
AAA33339	pectate lyase A [*Fusarium solani* f. sp. *pisi*]	70/240 (29%)	2e-10
AAC49420	pectate lyase D [*Fusarium solani* f. sp. *pisi*]	54/159 (33%)	6e-09

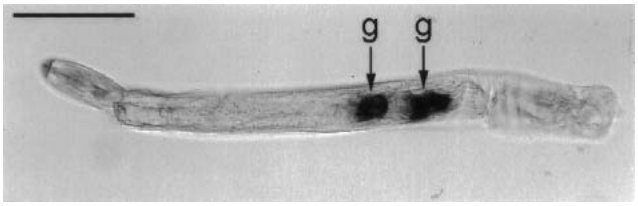

FIG. 2. Detection (dark staining) of *Hg-pel-1* mRNA in the subventral gland cells (g) of a second-stage juvenile of *H. glycines* by in situ hybridization. Scale bar = 50 μm.

they belonged to the same mRNA. The largest cDNA fragment had a poly-A tail of 19 nucleotides at the 3′ end and encompassed all of the six shorter fragments. The sequence of this largest cDNA fragment was submitted to GenBank under accession AY026357. The corresponding gene was named *Hg-pel-1*.

The deduced amino acid sequence of the *Hg-pel-1* cDNA fragment shows 72% identity (Fig. 1) with the deduced amino acid sequence of *Gr-pel-1* (Popeijus et al., 2000). In contrast to GR-PEL-1 protein, the HG-PEL-1 protein sequence has a consensus N-terminal glycosylation site located at Asp-23. An incomplete signal peptide of 16 residues was predicted in HG-PEL-1 by SignalP analysis. The close alignment of this signal peptide with the GR-PEL-1 signal peptide may suggest that only the first two amino acids are missing from the HG-PEL-1 protein sequence. Sequence database searches showed that, similar to GR-PEL-1, the HG-PEL-1 protein sequence has homology to bacterial and fungal pectate lyases of the class III type (Table 1).

In situ hybridizations to J2 of *H. glycines* with an antisense RNA probe revealed that *Hg-pel-1* is transcribed in the subventral esophageal gland cells (Fig. 2). No hybridization signals were obtained when the mRNA in the J2 tissues was degraded with RNase A prior to hybridization with the antisense probe, or if the J2 were hybridized with the sense probe.

The cloning of an esophageal gland cell cDNA from *H. glycines* with sequence similarity to a pectate lyase of *G. rostochiensis* suggests that, like EGases, pectin-degrading enzymes also may have a wide distribution among endoparasitic nematodes. The predicted N-terminal signal sequence in HG-PEL-1 indicates that this protein is secreted from the subventral gland cells. Further experiments are needed to determine whether expression of *Hg-pel-1* is developmentally regulated and whether this gene indeed encodes a pectate lyases that is secreted from the esophageal gland cells.

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