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Molecular Characterization of a Soybean Cyst Nematode (*Heterodera glycines*) Homolog of *unc-87*

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Abstract: In *Caenorhabditis elegans* the *unc-87* gene encodes a protein that binds to actin at the I band and is important in nematodes for maintenance of the body-wall muscle. *Caenorhabditis elegans* mutant phenotypes of *unc-87* exhibit severe paralysis in larvae and limp paralysis in the adult. We cloned and characterized a full-length cDNA representing a *Heterodera glycines* homolog of the *unc-87* gene from *C. elegans* that encodes a protein that contains a region of seven repeats similar to CLIK-23 from *C-elegans* and has 81% amino acid identity with that of *C. elegans unc-87* variant A. In the EST database clones labeled ''*unc-87*'' encode mainly the 3′ portion of *unc-87,* while clones labeled ''calponin homolog OV9M'' contain mainly DNA sequence representing the 5′ and middle transcribed regions of *unc-87.* A 1770 nucleotide cDNA encoding *H. glycines unc-87* was cloned and encodes a predicted UNC-87 protein product of 375 amino acids. The expression of *unc-87* was determined using RT-PCR and, in comparison to its expression in eggs, *unc-87* was expressed 6-fold higher in J2 juveniles and 20-fold and 13-fold ($P = 0.05$) higher in nematodes 15 and 30 days after inoculation, respectively. In situ hybridization patterns confirmed the expression patterns observed with RT-PCR.

Key words: gene expression, *Heterodera glycines,* in situ hybridization, soybean cyst nematode, *Unc-87.*

In *Caenorhabditis elegans* numerous genes have been associated with movement and a number of movement mutants have been identified and studied. One class of movement mutants demonstrates an *unc*oordinated phenotype and is known as *unc* mutants. A collection of *unc* mutants having disorganized muscle structure was first described by Waterston et al. (1980). Currently there are 114 members in the *unc* class of mutants (Johnson and Ballie, 1997). One gene, *unc-87,* is required for maintaining the body-wall muscle of the nematode, and mutants of *unc-87* demonstrate severe paralysis in the larva and limp paralysis in the adult (Moerman and Fire, 1997). In *C. elegans unc-87* is not considered an essential gene, possibly because movement is not required for its survival on a petri plate containing bacteria as a food source. However, it may be essential to *H. glycines,* which is required to move to a host to feed and reproduce. The *C. elegans* protein product, UNC-87, is related to calponin, an actin binding protein. Calponin contains a unique, repeated motif of 23 amino acids, known as the CLIK-23 repeat, and is located toward the C-terminal region of the protein. There is evidence that the CLIK-23 motif binds to actin (Gimona and Mital, 1998) and the protein is involved in actin bundling (Mino et al., 1998). In *C. elegans* UNC-87 contains seven CLIK-23 repeats.

The major pest of soybean is the soybean cyst nematode (SCN), *Heterodera glycines.* Over the past 5 years, estimated yield losses have ranged between 142 million and 279 million bushels, reflecting $0.7 to $1.5 billion in lost revenue to the farmers in the United States (Wrather et al., 2001). SCN is an obligate endoparasite that feeds in soybean roots, causing growth suppression, leaf chlorosis, and root stunting and necrosis. Each female can produce 200 to 600 eggs and the nematode can pass through several reproductive cycles during a growing season. After hatching the nematode uses an undulating motion to migrate from the site of hatching to the soybean root, where it burrows into the root and selects a cell near vascular tissue to begin feeding. Because locomotion and muscle contraction are essential to its survival, we hypothesize that *unc-87* may be required for one or both of these functions in SCN. Consequently, silencing of this gene may disrupt movement of SCN to and into the root, making *unc-87* a possible target for nematode control.

In this paper we describe a cDNA from *H. glycines* that is homologous to *C. elegans unc-87* and demonstrate its expression at several stages of *H. glycines* development.

Materials and Methods

cDNA sequence and analysis: Heterodera glycines EST clones RO41, RO55, and RO58 (Genbank accession numbers BG310718, BI451525, BI451722, respectively) were derived from a cDNA library constructed using a soybean cyst nematode inbred line (Dong and Opperman, 1997) and were obtained from Washington University Genome Sequencing Center, St. Louis, Missouri. Polymerase chain reaction (PCR) was used to obtain 5′ ends of truncated gene family members using the directionally cloned SCN library from E.L. Davis (North Carolina State University) as template and a forward primer, T3, based on vector sequence and reverse primers near the 5′ end of known cloned sequence. A full-length cDNA clone was amplified using forward primer CTCTCCCGTGAGCATCAATC located 110bp upstream of the methionine start site and reverse primer CACTCTCAACATTTATCATTTC located just before the poly A tail. The PCR products were gel purified and

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introduced into pCR4-TOPO II vector according to manufacturer's instructions (Invitrogen, Carlsbad, CA). DNA sequences were obtained using the ABI Big Dye Terminator Cycle Sequencing Kit and the ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Applied Biosystems, Foster City, CA). All clones were sequenced in both directions and assembled using Lasergene software (DNASTAR Inc, Madison, WI).

Nematode material and plant inoculation: The SCN population NL1-RH was originally collected from Eastern Shore, Maryland, interacts with soybean differentials in the manner of race 3, and can be obtained from the SCN Stock Center (T.L. Niblack, University of Illinois, Champaign Urbana, IL). Nematodes were harvested at various stages including egg, J2, and 15 days and 30 days post-inoculation, as previously described (Matthews et al., 2003) by harvesting cysts using the procedure of Krusberg et al. (1994) and purifying the cysts by centrifugal flotation (Barker, 1985; Jenkins, 1964). Two-week-old soybean plants were maintained and inoculated as described (Matthews et al., 2003) using 2,000 J2/seedling. Nematode invasion and development were monitored by fuchsin staining (Byrd et al., 1983).

Computer analysis: Nucleotide and protein sequences were compared using BLAST tools (Altschul et al., 1997). Clones with high amino acid (aa) sequence identity to *unc-87* sequences were obtained from the SCN EST database and used to generate contigs to determine family members and relationships. Contigs were generated using Lasergene software. Dendrograms were generated using Genetics Computer Group software programs (Accelyrs, San Diego, CA) Pileup, Distance, and Growtree using the Tahim-Nei algorithm (Nei et al., 1983) for nucleotide sequences and the Kimura algorithm for peptide sequences.

qRT-PCR: RNA was extracted from nematodes isolated at different stages using the methods of Mujer et al. (1996). Genomic DNA was removed by treatment with DNase I. The RNA was reverse transcribed into cDNA using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, NY) and oligo d(T) as primer according to manufacturer's instructions. Genomic DNA contamination was assessed by PCR using *unc-87* PCR primers that amplify different size fragments in the presence of genomic DNA compared to cDNA, specifically, forward primer GACAACACGGAGATTCCACTTCAG and reverse primer CTGGTCTGGTCGATGCTCTGCTC. Relative quantities of *unc-87* and 28S rRNA (forward primer GGAGCGCACGAGACACCACA; reverse primer CGGTAACGCAAATGAACTCG) were determined using a Stratagene (La Jolla, CA) Mx3000P Real-Time PCR system following the manufacturer's instructions. SYBR green was used to measure DNA accumulation, and ROX was used as reference dye. RT-PCR containing primers to the reference 28S rRNA were used in parallel with reactions containing dilutions over a five-log range. Controls for RT-PCR included reactions containing no template, and reactions using RNA that were processed in parallel but with no Superscript reverse transcriptase. Assays were conducted in triplicate. Threshold cycle (C_t) values were plotted against the dilution series. PCR efficiencies were equal between the target and endogenous control. C_t values and relative abundance were calculated using software supplied with the Mx3000P Real-Time PCR system. Fold change was calculated by comparing the relative abundance of *unc-87* mRNA in the different sample types to 28S RNA. The amount of mRNA for the different stages was analyzed in an analysis of variance, and means were compared with least significant difference ($P = 0.05$). The SYBR green dissociation curve of amplified products demonstrated the production of only one product. Terminal RT-PCR products were assayed by gel electrophoresis in 1% agarose to ensure that only one product was produced and that the product was of proper size. For RT-PCR, the DNA was denatured for 10 minutes at 96 °C. Then PCR cycling times and temperatures were 30 seconds at 96 °C, 1 minute at 55 °C, and 30 seconds at 72 °C.

Plant and nematode tissue preparation: Glycine max cv. Kent roots were infected with *Heterodera glycines* race 3 and allowed to grow at ambient greenhouse temperatures for 8 days. The nematode-infected roots were washed with distilled water and cut into 0.5-cm sections with a fresh razor blade. The root fragments were killed and fixed under vacuum for 15 minutes in a 75% ethanol, 25% glacial acetic acid (Farmer's fixative) solution (Sass, 1958) with 0.1% acid fuschin to visualize the root pieces. The vacuum was released slowly, and the root specimens sank to the bottom of the glass vial. The fixative solution was replaced with fresh fixative and the specimens were incubated at 4 °C overnight. Dehydration of the specimens occurred through 30-minute incubations in 75% and 85% ethanol made with diethylpyrocarbonate (DEPC)-treated dH_2O. Dehydration proceeded through two changes of 100% ethanol for 30 minutes each. The ethanol was replaced by the sequential addition of 25%, 50%, and 75% xylene for 3 hours in each step. The specimens were subjected to three changes of 100% xylene and then placed into a 58 °C incubator for 30 minutes. Infiltration was performed by the sequential addition of melted Paraplast Plus Tissue Embedding Medium (Oxford Labware, St. Louis, MO) to approximate proportions of 25%, 50%, and 75% xylene:paraplast for 3 hours in each step. The paraffin was replaced by three changes of 100% Paraplast Plus for 3 hours in each step. The specimens were oriented in specimen boats on a hot plate set at 58 °C. The Paraplast Plust was slowly cooled to permanently embed the tissue samples. The tissue was stored at 4 °C. For pre-parasitic nematodes and mature females, the living samples were placed into 4% paraformaldehyde in M9 salts (Brenner, 1971), treated identically as de-

scribed for the root specimens with the exception that the preparasitic J2 and mature female nematodes were solidified in Paraplast Plus in 2-ml microcentrifuge tubes. Tissue samples were excised from the solidified paraffin and mounted onto wood blocks. Samples were cut on an American Optical 820 microtome (American Optical Co., Southbridge, MA) at a section thickness of 15 to 20 µm. The sections were placed onto a pool of DEPC-treated water on baked RNAse-free slides coated previously with polylysine according to the manufacturer's instructions (Sigma, St. Louis, MO). The sections were warmed to 42 °C on a slide warmer to spread the sections. Excess DEPC water was removed via blotting with a Kimwipe (Kimberly-Clark Corp., Mississauga, Ontario). The slides were removed from the slide warmer once the DEPC water had evaporated and used for in situ hybridization.

Probe generation. Portions of *Hg-Unc-87* and the cDNA encoding arginine kinase (Matthews et al., 2003) were amplified by PCR. The endogenous SP6 promoter of pAMP1 that carried these sequences was removed via PCR. The SP6 promoter removal was accomplished by creating a PCR primer that incorporated a T3 promoter sequence downstream of the SP6 promoter. This T3 sequence was followed by a nested pAMP1 vector sequence. PCR amplification occurred with the primers pAMP1 T3 5′ → 3′ CGAAATTAACCCTCACTAAAGGGGCTTGGATCCTCT AGAGCGGCCGC and pAMP1 T7 5′ → 3′ TGTAATACGACTCACTATAGGGAAA GCTGGTACGCCTGCAGGTACCG with the following conditions: denaturation, 96 °C for 30 seconds; annealing, 50 °C for 15 seconds; extension, 60 °C for 4 minutes (30 cycles) using Platinum *Taq* (Invitrogen, Grand Island, NY) to generate DNA sequences flanked by T3 and T7 promoters for production of a portion of *Unc-87* and a portion of the sequence encoding arginine kinase. The PCR inserts were gel purified according to the manufacturer's instructions (Qiagen, Valencia, CA).

In vitro transcriptions were performed according to the manufacturer's instructions (Epicentre, Madison, WI) with the exception that digoxigenin-11-UTP (Roche Diagnostics Corp., Basel, Switzerland) was added. Briefly, 1 µg of DNA template was added to a 10-µl transcription reaction. The reaction consisted of 7.5 mM ATP, CTP, GTP, and 0.25 mM dUTP, 2.5 mM digoxigenin-11-UTP, 10 mM dithiothreitol (DTT), 1X reaction buffer, 1X T3 or T7 polymerase. The reaction proceeded at 37 °C for 3 hours in a PCR machine. RNA was NH_4OAC precipitated to remove unincorporated nucleotides. The RNA probe was re-suspended in 40 µl of 50% deionized formamide. Then the RNA probe was denatured at 80 °C for 2 minutes followed by incubation on ice for 5 minutes. The riboprobe was diluted into hybridization solution [HS; 10% in situ salts (3 M NaCl, 100 mM Tris pH 8.0, 100 mM Na phosphate pH 6.8, 50 mM EDTA), 40% deionized formamide, 20% of a 50% dextran sulfate, 2% 50X Denhardt's solution, 1% 100 mg/ml tRNA, 7% DEPC dH_2O] to bring the final riboprobe concentration in HS to 50 to 100 ng/slide. Then, 160 µl of hybridization solution [HS; 10% in situ salts (3 M NaCl, 100 mM Tris pH 8.0, 100 mM Na phosphate pH 6.8, 50 mM EDTA), 40% deionized formamide, 20% of a 50% dextran sulfate, 2% 50X Denhardt's solution, 1% 100 mg/ml tRNA, 7% DEPC dH_2O] was added to this to bring the final hybridization volume to 200 µl. Then, 200 µl of probe solution was added per slide at a riboprobe concentration of 50 to 100 ng/slide.

In situ Hybridization. The in situ hybridization protocol was adapted from Long et al. (1996) and the Barton Lab Web page (http:carnegiedpb.stanford.edu/research/barton/in_situ_protocol.html). All solutions were made RNAse-free by the addition of DEPC, and all glassware was baked at 200 °C overnight. In brief, slides were washed at ambient temperature in 100% xylene (2 X 10 minutes), 1:1 xylene:ethanol (ETOH) (1 X 1 minute); 100% ethanol (2 X 1 minute), 95% ETOH (1 X 1 minute), 90% ETOH (1 X 1 minute), 80% ETOH (1 X 1 minute), 60% ETOH (1 X 1 minute), 30% ETOH (1 X 1 minute), dH2O (1 X 1 minute), and 2X sodium citrate (SSC) buffer (15 minutes). After washing, the tissue was subjected to a proteinase K (1 µg/ml; Boehringer Mannheim Corp., Indianapolis, IN) treatment at 37 °C (30 minutes). Proteinase treatment was halted by incubation in glycine (2 mg/ml) in 1X PBS (2 minutes) followed by two 1-minute washes in PBS. Post fixation was accomplished with a 4% paraformaldehyde (in PBS pH 7.0) treatment for 10 minutes followed by two 1-minute washes in PBS. Elimination of potential non-specific probe binding was accomplished by acetylating the tissue with a 10-minute wash in 0.1 M triethanolamine pH 8.0 with the addition of 5 mM acetic anhydride. Slides were washed in PBS (2 X 5 minutes) followed by successive dehydration steps through 30%, 60%, 80%, 90%, and 95% ETOH (1 X 30 seconds), finishing with 100% ETOH (2 X 30 seconds washes). The slides were then air dried on paper towels. RNA probes of *Hg-Unc-87* and *Hg-AK-1* were dropped onto the slides and covered with parafilm. The slides were placed in a humid box where the hybridization took place overnight at 55 °C. After hybridization, slides were washed twice with 0.2X SSC for 60 minutes at 55 °C. The slides then were transferred to and washed twice in NTE (10 mM Tris, pH 8.0, 0.5 M NaCl, 1.0 M EDTA) for 5 minutes at 37 °C. To eliminate residual single-stranded riboprobe, the slides were subjected to an RNAse A (Boehringer Mannheim, Indianapolis, IN) treatment (20 mg/ml in NTE) for 30 minutes at 37 °C. The slides were transferred through two 5-minute NTE washes at 37 °C followed by a 60-minute 0.2 X SSC wash at 55 °C. The slides were transferred to 1 X PBS for 5 minutes at ambient temperature and then placed in a humid chamber with 500 µl of 1% blocking solution (Boehringer) in 100 mM Tris pH 7.5, 150 mM NaCl

and incubated for 45 minutes. The blocking solution was then replaced with 1% BSA, 100 mM Tris pH. 7.5, 150 mM NaCl, and 0.3% Triton X-100 for 45 minutes. The solution was blotted off and the slides were repositioned in the humid chamber. Anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) was diluted 1:1,250 in the BSA solution. Diluted antibody solution (300 µl) was placed on each slide and incubated at ambient temperature for 2 hours. The antibody solution was blotted off and the slides were washed for 15 minutes four times in BSA solution. The slides were blotted dry and were washed once in 100 mM Tris (pH. 9.5), 100 mM NaCl and 50 mM MgCl2 for 10 minutes. The slides were then dipped in fresh Tris pH 9.5 wash solution. To detect for the presence of the digoxigenin, NBT/BCIP (Boehringer) solution was diluted 1:5 in the Tris pH 9.5 wash solution and added 400 µl/slide. The solution was immediately blotted from the slides and replaced with fresh NBT/BCIP solution. The slides were placed in total darkness and the color reaction was allowed to progress until ample color detection had occurred. The reaction was stopped by placing the slides in TE. The slides were washed in dH_2O (2 X 5 minutes) and semi-permanently mounted.

Results

Characterization of three unc-87 *cDNAs:* There are nine EST sequences in the *H. glycines* EST database listed as *unc-87* (Table 1). These clones were derived from cDNA libraries made from *H. glycines* eggs, J2, and virgin females. We obtained and resequenced four of these clones, which were provided by the *H. glycines* EST sequencing project (Wylie et al., 2004; See: Nematode.Net, Genome Sequencing Center (*http://www.nematode.net/*), as indicated in Table 1. The amino acid sequence of the proteins encoded by three of these clones proved to be similar to a gene encoded by *unc-87,* variant A, of *C. elegans.* The fourth clone was determined to be a truncated version of one of the other 3 clones and was eliminated from further analysis. PCR primers were designed from these DNA sequences to amplify the 5′ and 3′ ends of the coding regions of the genes from an *H. glycines* J2 cDNA library. These amplification products were sequenced, aligned, and assembled to provide a cDNA sequence that retained extensive amino acid identity with that encoded by *C. elegans unc-87.* Although there was a methionine present that could be a putative translation start site, the amino acid sequence 5′ of the methionine continued to retain significant amino acid identity, suggesting that the start site was further upstream and that there was 5′ sequence missing. Therefore, a portion of the DNA sequence at the 5′ end of our longest clone was compared to the EST database, and more than 90 calponin-homolog-OV9M clones were identified with a similar sequence. Alignment of these clones provided an additional 5′ sequence that encoded a methionine corresponding to the methionine start site of *C. elegans unc-87* variant A.

Table 1. *H. glycines* EST representing *unc-87.*

Genbank Accession No.	Plate address	Source
CB380327	rq69a09	egg
CB375193	rq98f07	virgin female
CB374199	rq58d04	egg
CA940182	rq46d03	egg
B1749102	ro73h06	J2
BI451722[1]	ro58c12	J2
BI451525[1]	ro55g07	J2
BI396517[1]	ro54a01	J2
BG310718[1]	ro41g11	J2

[1] Indicates that the clone was obtained and the insert sequenced.

PCR primers were designed to amplify the full-length cDNA sequence of *H. glycines unc-87* for cloning. The PCR-amplified sequence of 1770 nt contained an open reading frame of 1125 nt encoding a predicted protein of 375 aa (Fig. 1) The resulting protein has a predicted molecular mass of 41.7 kDa and a theoretical isoelectric point of 7.93.

Comparison of Hg-unc-87 *with C. elegans* unc-87 *variant A.* The deduced amino acid sequence of *Hg-UNC-87* has 81% identity to *C. elegans* UNC-87. It contains seven repeated regions of 23 aa that have high similarity (%) to the CLIK-23 repeat, also found seven times in UNC-87 of *C. elegans* (Fig. 2).

Expression of Hg-unc 87. Primers were designed for specific amplification of the Hg-unc-87 gene for measurement of expression by RT-PCR and SCN 28S rRNA, as a control RNA. When these *unc-87* primers were used in the presence of cDNA, the product size was smaller than when genomic DNA was used as template, indicating that these primers can be used to assay for genomic DNA contamination of cDNA.

To determine at which developmental stage the *unc-87* gene is most highly expressed, RT-PCR was performed on SCN eggs, J2, and nematodes obtained 15 days post-inoculation of the soybean root, and on mature females at 30 days post-inoculation (Fig. 3). Expression of *unc-87* in J2, which require the ability to move to the root and burrow into the root, was 6-fold higher than in eggs, 150-fold higher than in nematodes 15-days post-inoculation, and 300-fold higher than in nematodes 30 days post-inoculation.

In situ hybridizations. We performed in situ hybridizations on paraffin-embedded plant tissues that had been infected with *H. glycines,* and also on pre-parasitic nematodes. Both complementary antisense (Fig. 4a–d) and control sense (Fig. 4e–h) digoxigenin-labeled RNA probes for *Hg-Unc-87* were used to confirm the RT-PCR observations that *Hg-Unc-87* is present at various stages during the life cycle of *H. glycines.* We also performed, simultaneously, in situ hybridizations for complemen-

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TCTCCCGTGAGCAGCAATCAGCAAAAGCTGAACACACTCCTTCGAACTCACTGATTATTT      60
S  P  V  S  S  N  Q  Q  K  L  N  T  L  L  R  T  H  *  L  F
GATCCATCTCTGAGAGCAACTTCTGATCAGCCAAAGATGCCGGAAAGCGAAAACGACTTC    120
D  P  S  L  R  A  T  S  D  Q  P  K  M  P  E  S  E  N  D  F
GATGAGGAGGCGCCCAACGCCACCATGGAGACCAAAGTGGCGGGCGGCGGCGCGGCGCCG    180
D  E  E  A  P  N  A  T  M  E  T  K  V  A  G  G  G  A  A  P
AAGCGCGTCGGGCGCTGGACGTTGTCACAGCTGCGCCAGACGGACGGCATCATCCCGTCC    240
K  R  V  G  R  W  T  L  S  Q  L  R  Q  T  D  G  I  I  P  S
CAGGCCGGTTGGAACAAGGGAGACTCGCAGAAGTTGATGACCAACTTTGGTACGCCGCGT    300
Q  A  G  W  N  K  G  D  S  Q  K  L  M  T  N  F  G  T  P  R
AACACCACCACCAAAGTCAAAGTGGAGAATCTGGCCGAGATTCCGGAGGACATTTTGCTG    360
N  T  T  T  K  V  K  V  E  N  L  A  E  I  P  E  D  I  L  L
AAAAGCCACGGGGAGGTGCGTCTGCAGTCCGGTACCAACCGGTTCGCCTCCCAGAAGGGC    420
K  S  H  G  E  V  R  L  Q  S  G  T  N  R  F  A  S  Q  K  G
TTTGTTTCGTTCGGTACCGGACGTGATGTGTGTCGTGAAGGTGTGAATGTGAACATCCTG    480
F  V  S  F  G  T  G  R  D  V  C  R  E  G  V  N  V  N  I  L
CCGGCCGACTTGGAGCCGCTGCCGGAGGAGAAAATCCGCGCCAGTGACGGCATTGTGCGT    540
P  A  D  L  E  P  L  P  E  E  K  I  R  A  S  D  G  I  V  R
CTGCAAGCCGGTACGAACAAATTCGACTCGCAGAAGGGCATGACCCTGTTCGGTACCGGA    600
L  Q  A  G  T  N  K  F  D  S  Q  K  G  M  T  L  F  G  T  G
CGTCGTGAGACCACCAGAATGAAGGACAGCAAGCACCCGGAGTACTCGCACGAGCGCGAC    660
R  R  E  T  T  R  M  K  D  S  K  H  P  E  Y  S  H  E  R  D
   ----------------------->
ATTGACAACACGGAGATTCCGCTTCAGATGGGTACGAACAAATTCGCCTCGCAGAAGGGC    720
I  D  N  T  E  I  P  L  Q  M  G  T  N  K  F  A  S  Q  K  G
ATGACTGGCTTCGGCACAAACCGTCGTGAGACCACCAAAATGTTGGACACCTCGCACCCG    780
M  T  G  F  G  T  N  R  R  E  T  T  K  M  L  D  T  S  H  P
            <-----------------------
GAGTACAGCCACGAGCAGAGCATCGACCAGACCAGCATCCCCTACCAAATGGGATCGAAC    840
E  Y  S  H  E  Q  S  I  D  Q  T  S  I  P  Y  Q  M  G  S  N
AAATACGCGTCGCAGAAGGGCATGACCGGCTTTGGACAGCCACGTTGGGAGGTGCTTGAC    900
K  Y  A  S  Q  K  G  M  T  G  F  G  Q  P  R  W  E  V  L  D
CCGTCCATTTCGTACCAGAACCGCAAGTCGCAAGGCATGGTTCGTCTCCAATCCGGCACC    960
P  S  I  S  Y  Q  N  R  K  S  Q  G  M  V  R  L  Q  S  G  T
AACCGATTCGCCTCCCAGGCGGGCATGACCGGCTTTGGCACGCCGAGGAACAACACCTAC   1020
N  R  F  A  S  Q  A  G  M  T  G  F  G  T  P  R  N  N  T  Y
GAGGCGGAGGCGGGCGAGCTGCCTTACGAGGATATGAAGAAGTCGGAGGCGATCATCCCG   1080
E  A  E  A  G  E  L  P  Y  E  D  M  K  K  S  E  A  I  I  P
TCCCAGGCCGGATGGAACAAGGGCGACTCGCAGAAGCTGATGACCAACTTTGGCACACCG   1140
S  Q  A  G  W  N  K  G  D  S  Q  K  L  M  T  N  F  G  T  P
CGTGACGTGAAGGGCAAACATTTGAAGCGCATTTGGGAATTGGAGTACCCGGAGGAAGCC   1200
R  D  V  K  G  K  H  L  K  R  I  W  E  L  E  Y  P  E  E  A
GAAGTGTCGCTGGACCGTCTCTGATCAGCGACACGGATTGAATTGTGCTGCTGATCAGCA   1260
E  V  S  L  D  R  L  *
CATGAATACACACGGAGGAAGGAGGAGTAGGACAAATGCCATCGTCCTCATCAAGTTGGA   1320
GCAGTACACACCCACCACACCGTCATCCTTTGCCGTCCGGAGAGTGATCGATCCATTGTT   1380
TGATAGATTGATTGATCAGAAAACATCCATCCATCCTCCGATCACAGCCCCTGGCATTTT   1440
TTGCTGCTTAAAACTTTGTGGTTTTAAATACATTTAGTGCCTGCGGCAACACTTTTTTTC   1500
AAATTTTTGCCTAATATTTCCTTCCCCTTTTTCTCTACCAACTCCATCAATCACACACTA   1560
TTTCGCTGCTCCGACTTTCCCTCCCCTCGCCCCATTTTTCCTCTCGGCTTCTCCCACCCC   1620
CAATTTTTTGTGCTTTAACGATACAAAAATATTATTAATCACTGAGATGAATGAATGGTT   1680
TGGATTTTCAATTCGGCTCACCACACATTTGCAAAATAATGGCGCGAAATGATAAATGTT   1740
GAGAGTGAAAAAAAAAAAAAAAAAAAAAAA                                 1770
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FIG. 1. DNA sequence and amino acid translation of *unc-87* of *Heterodera glycines* (accession number AY672636). The ATG start site, initiation methionine, and the stop codon, TGA, are presented in bold letters. Primers for the 5′ and 3′ ends of the RT-PCR are provided with an arrow above the nucleotide sequence. The *unc-87* DNA sequence was provided to Gen Bank under accession number AY 672636.

tary antisense (Fig. 4i–l) and control sense (Fig. 4m–p) Hg-Arginine Kinase (*Hg-AK1*) RNA. As an additional control, designed to observe the extent of color substrate alone that reacted to our tissues, we incubated the NBT color substrate solution with tissues that had no probe hybridized to them (Figures 4q–t). We observed that some *Hg-Unc-87* probe hybridized with the eggs of gravid females (Fig. 4a), J1-, and J2-stage nematodes before hatching from the egg (pre-emergent) (Fig. 4b). Also, expression was observed in pre-parasitic

H. glycines	I P S Q A G W N K G D S Q K L M T N F G T P R
	V R L Q S G T N R F A S Q K G F V S F G T G R
	V R L Q A G T N K F D S Q K G M T L F G T G R
	I P L Q M G T N K F A S Q K G M T G F G T N R
	I P Y Q M G S N K Y A S Q K G M T G F G Q P R
	V R L Q S G T N R F A S Q A G M T G F T G T P
	I P S Q A G W N K G D S Q K L M T N F G T P R
C. elegans consensus	I P L Q _ G T N K _ _ S Q K G M T G F G T _ R

FIG. 2. Comparison of the seven repeated sequences of *Heterodera glycines* aligned with the CLIK-23 consensus sequence of *Caenorhabditis elegans*.

stage nematodes (Fig. 4c). In nematodes introduced to plant tissues for 8 days, we observed some expression along the body-wall cavity, presumably along the somatic muscles (Fig. 4d). Minimal color formation was observed when using the sense-strand probe with eggs of gravid females (Fig. 4e), pre-emergent stage nematodes (Fig. 4f), J2 (Fig. 4g), and in nematodes that had penetrated plant tissue during an 8-day infection regime (Fig. 4h). Therefore, the hybridization reaction appears to be specific for the *Hg-Unc-87* transcript. Our comparisons of *Hg-Unc-87* expression profiles with *Hg-AK1,* a gene studied previously over the life cycle of the nematode (Matthews et al., 2003), showed that complementary labeled antisense *Hg-AK1* riboprobe hybridized within the eggs of gravid females (Fig. 4i), pre-emergent stage nematodes (Fig. 4j), J2 (Fig. 4k), and also within nematodes that had penetrated plants (Fig. 4l). Control sense labeled *Hg-AK1* transcripts yielded no hybridizations to eggs within gravid females (Fig. 4m). However, the eggs appeared dark from the yolk granules within the eggs and not from any riboprobe hybridization because both control eggs without digoxigenin probes (see Fig. 4q) and *Hg-Unc-87* sense controls (see Fig. 4e) also exhibited a similar dark appearance. Pre-emergent stage nematodes (Fig. 4n), J2 (Fig. 4o), and adults (Fig. 4p) did not exhibit any labeling. As expected, incubating specimens with the NBT color substrate solution alone yielded no reactivity with eggs within gravid females (Fig. 4q), pre-emergent stage nematodes (Fig. 4r), J2 (Fig. 4s), or within nematodes that had infected plants (Fig. 4t).

DISCUSSION

In *C. elegans,* the *unc-87* gene encodes a protein associated with thin filaments of the musculature. Mutations in the *unc-87* gene cause variable paralysis due to severe reduction in muscle function. The *C. elegans*

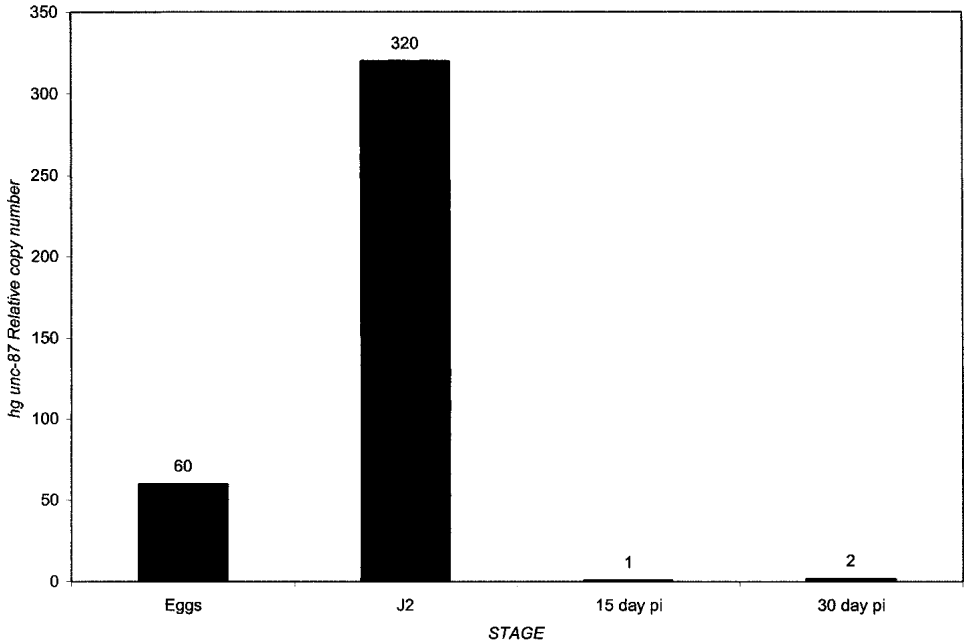

FIG. 3. Relative expression of *unc-87* in *Heterodera glycines* eggs, J2, and females 15 days and 30 days post-inoculation as compared to expression of 28S rRNA. Assays were conducted in triplicate.

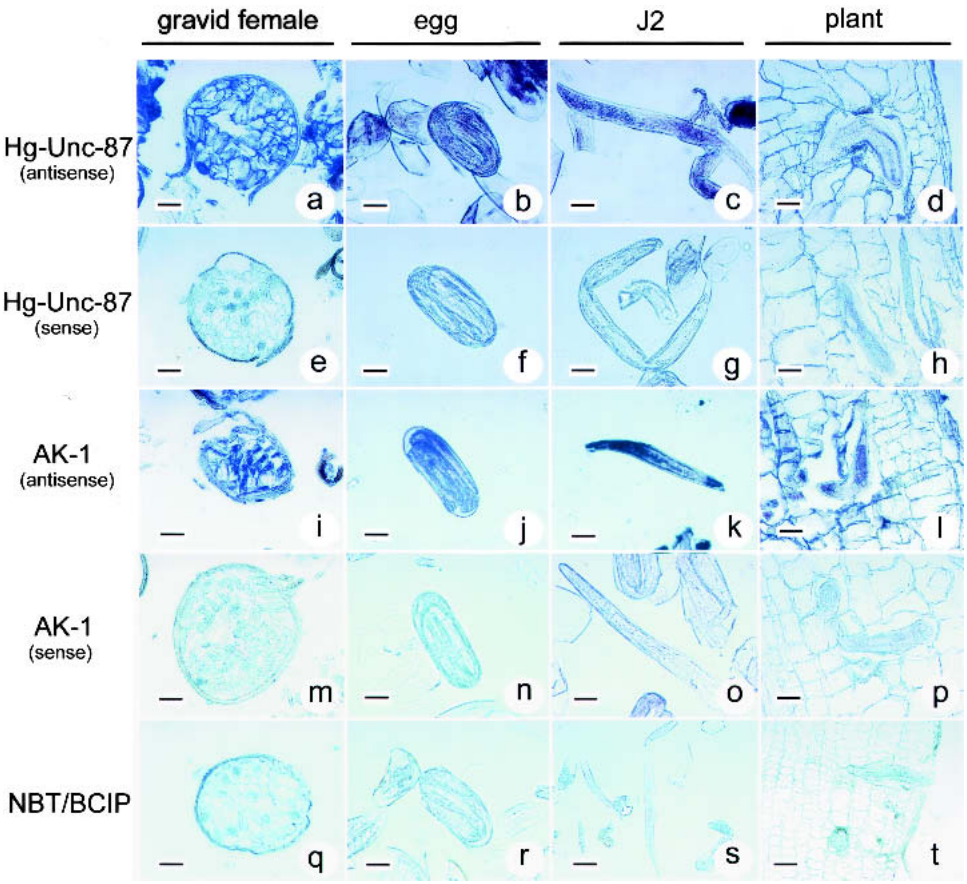

FIG. 4. In situ hybridizations were performed on cross sections of paraffin-embedded plant tissues infected with *Heterodera glycines* nematodes with both complementary Hg-Unc-87 antisense (Fig. 4a–d) and control sense (Fig. 4e–h) digoxigenin-labeled RNA probes, complementary antisense Hg-AK1 (Fig. 4i–l) and control sense (Fig. 4m–p) RNA probes, and probe-less specimens with the NBT color substrate solution (Fig. 4q–t). a) Gravid female with antisense Hg-Unc-87 (bar = 100 μM). b) Pre-emergent juvenile with antisense Hg-Unc-87 (bar = 25 μM). c) Pre-parasitic J2 with antisense Hg-Unc-87 (bar = 50 μM). d) Nematode penetrating plant root at 8 days with antisense Hg-Unc-87 (bar = 50 μM). e) Gravid female with sense Hg-Unc-87 (bar = 100 μM). f) Pre-emergent stage nematode with sense Hg-Unc-87 (bar = 25 μM). g) Pre-parasitic J2 with sense Hg-Unc-87 (bar = 50 μM). h) Nematode penetrating plant root at 8 days with sense Hg-Unc-87 (bar = 50 μM). i) gravid female with antisense Hg-AK1 (bar = 100 μM). j) Pre-emergent stage nematode with antisense Hg-AK1 (bar = 25 μM). k) Pre-parasitic J2 with antisense Hg-AK1 (bar = 50 μM). l) Nematode penetrating plant root at 8 days with complementary antisense Hg-AK1 (bar = 50 μM). m) Gravid female with sense Hg-AK1 (bar = 100 μM). n) Pre-emergent stage nematode with sense Hg-AK1 (bar = 25 μM). o) Stage J2 nematode with sense Hg-AK1 (bar = 50 μM). p) Nematode penetrating plant root at 8 days with complementary sense Hg-AK1 (bar = 50 μM). q) Gravid female incubated with NBT/BCIP reaction solution only (bar = 100 μM). r) Pre-emergent stage nematode incubated with NBT/BCIP reaction solution only (bar = 25 μM). s) Pre-parasitic J2 incubated with NBT/BCIP reaction solution only (bar = 50 μM). t) Nematodes that had penetrated plant tissue during an 8-day infection regime incubated with NBT/BCIP reaction solution only (bar = 100 μM).

UNC-87 protein is associated with thin filaments of striated muscle, a predominant muscle in the body wall that is involved in body movement. UNC-87 contains seven CLIK-23 repeats (Goetinck and Waterston, 1994a,b); these repeats bind actin and UNC-87, which is involved in actin bundling (Kranewitter et al., 2001). In living cells, UNC-87 promotes the formation of actin stress fiber bundles (Kranewitter et al., 2001).

Here we describe the *unc-87* gene from *H. glycines,* which has features of the *unc-87* gene of *C. elegans,* including seven tandem CLIK-23 repeats in the protein. Expression of *unc-87* is high in J2, which require body movement to move to roots and to burrow into the roots. The sedentary nematodes seen at 15 and 30 days post-inoculation have greatly reduced (more than 150-fold less) levels of *unc-87* transcript than the J2 stage. The *unc-87* gene is expressed over 5-fold less in eggs than in J2. The eggs may contain early embryos and J1, which are non-motile, or may contain J2 (motile) nematodes that will require movement for hatching and migration into the soybean root. The eggs have 30-fold more *unc-87* transcript than the sedentary nematode at 15 and 30 days post-inoculation.

A calponin-like gene (*unc-87*) was previously described from the root knot nematode, *Meloidogyne incognita* (Castagnone-Sereno et al., 2001). The encoded protein contained seven highly conserved, repeated regions similar to CLIK-23. Expression of this gene was detected readily in J2 and males but was not detected in eggs and in females (the time of post-inoculation was not given). Males and J2 are motile and would require the expression of *unc-87* for movement, while the fe-

males were non-motile. Similarly, we found *unc-87* were most highly expressed in J2-stage nematodes, and expression levels in female nematodes at 15 days and 30 days post-inoculation were 150- and 300-fold lower.

To confirm the RT-PCR results for the presence of *Hg-Unc-87* RNA in nematodes, we performed in situ hybridizations to gravid females, eggs, J2, and nematodes that had invaded roots during an 8-day infection regime. As anticipated from our RT-PCR experiments, we observed some eggs within gravid females expressing limited Unc-87. In addition, we observed *Hg-Unc-87* expression in J2-stage nematodes, while *Hg-Unc-87* expression was limited to the somatic muscles in females becoming sessile. The observation that *Hg-Unc-87* is highly similar to *Ce-Unc-87* and that its mRNA is present within the body walls of the mobile J2 consistent with the protein being involved in body–wall architecture and movement (Goetnick and Waterston, 1994a). However, we have not yet confirmed this by immunological localization with anti-UNC-87 antibodies or other experimental conditions that would inhibit Hg-UNC-87 function. As the female nematode makes the transition from a motile nematode to a nonmotile, feeding parasite, the body cavity of the female nematode changes. The muscles deteriorate and the female becomes nonmotile as it establishes a feeding site within the root. Therefore, it is not surprising to observe a rapid decrease in the amount of *Unc-87* mRNA in late-stage *H. glycines* females as demonstrated both by RT-PCR and in situ hybridization.

Arginine kinase is a protein that provides ATP energy for muscles, and its expression pattern appears different from that of *Hg-unc-87*. Although arginine kinase has been implicated in restoring the ATP pool required to supply the energy for muscles in diverse organisms, this enzyme has not been shown to interact with UNC-87. The only protein shown biochemically to interact with and be phosphorylated by arginine kinase is actin (Reddy et al., 1992). Interestingly, UNC-87 has been shown to exhibit actin bundling properties (Kranewitter et al., 2001).

Whereas we have used *Hg-AK1* for comparison purposes in our experiments, we have no evidence that the proteins encoded by *Hg-Unc-87* and *Hg-AK1* interact directly in any way or that Hg-AK1 phosphorylates Hg-UNC-87. With respect to Hg-AK1, the observations here imply that Hg-AK1 may serve another function, as the body muscles (composed in part by UNC-87) degenerate in the females as they become nonmotile. Other possible roles for Hg-AK1 may include augmenting the establishment of the feeding apparatus that is composed in part of stylet protractor muscles, growth as the female increases in size during feeding, or egg production. These processes are all likely to be large energy sinks. Consistent with this idea is that arginine kinase has been shown to be involved in neural networking (Wang et al., 1998), thus confirming that arginine kinase plays biological roles other that its well-established muscular function. Conversely, our observations indicate that Hg-UNC-87 likely serves entirely as a body-wall muscle protein as previously characterized in *C. elegans* (Goetnick and Waterston, 1994a).

In *H. glycines* the *unc-87* gene is expressed in high levels in J2 and may be a good candidate for RNAi gene silencing studies, which presumably would yield an uncoordinated phenotype as seen in *C. elegans* mutants. If mobility of the nematode in the next generation could be compromised by the ingestion by female nematodes of RNAi produced by soybean roots and designed to silence *unc-87,* this may provide a new method of control of SCN.

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