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Molecular Characterization of Arginine Kinases in the Soybean Cyst Nematode (*Heterodera glycines*)[1]

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Abstract: Arginine kinase (AK) is a phosphagen kinase that plays a key role in energy mobilization in invertebrates. Alignment of expressed sequence tags (ESTs) for soybean cyst nematode (SCN) (*Heterodera glycines*) produced two separate contiguous sequences (contigs) and three singletons encoding peptides with high similarity to AKs. One contig, Hg-AK1, had 244 ESTs in the alignment whereas the other, Hg-AK2, had only three; nonetheless, the consensus sequence for Hg-AK1 was missing much of the 5′ end. Polymerase chain reaction (PCR) was used to prepare clones that were then sequenced to obtain full-length sequences for both Hg-AK1 and Hg-AK2. Hg-AK1 has an open reading frame of 1080 nucleotides (nt) encoding a protein of 360 amino acids (aa) with a predicted molecular weight of 40 kDa. The open reading frame for Hg-AK2 is 1221 nt, 407 aa, and 46 kDa with a 71% aa identity with Hg-AK1. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) indicated that Hg-AK1 and Hg-AK2 are expressed constitutively throughout the SCN life cycle. Phylogenetic analysis of peptide sequences for near full-length nematode contigs and other AKs in the Swisspro database indicates that the nematode AKs evolved from a single gene after divergence of insects and nematodes.

Key words: arginine kinase, gene expression, *Glycine max, Heterodera glycines,* soybean, soybean cyst nematode.

Phosphagen kinases play several roles in animals, including serving as a temporal and spatial energy buffer. In mammals, creatine kinase (CK) is the major phosphagen kinase and has been extensively studied in a number of vertebrate systems (Muhlebach et al., 1994; Suzuki and Furukohri, 1994). Four nuclear-encoded CK isoenzymes have been identified in different bird and mammalian tissues, including two isoenzymes found in the mitochondrion (Muhlebach et al., 1994). In invertebrates, arginine kinase (AK) (ATP:L-arginine *N*-phosphotransferase; EC 2.7.3.3) is the common phosphagen kinase and catalyzes the reversible, magnesium-dependent transphosphorylation: *N*-phospho-L-arginine + ADP ↔ ATP + L-arginine. Arginine kinases from numerous different invertebrates have been described (Suzuki et al., 1999), including several insects: fruit fly *Drosophila melanogaster* (Collier, 1990; Munneke and Collier, 1988), tobacco hornworm *Manduca sexta* (Chamberlin, 1997), locust *Lucusta migratoria* (Schneider et al., 1989), and honey bee *Apis mellifera* (Kucharski and Maleszka, 1998); mollusks: scallop *Pecten maximus* (Reddy et al., 1991), chiton *Liolophura japonica* (Suzuki et al., 1997a), and octopus *Octopus dofleini* (Zinovieva et al., 1999); and the insect parasitic nematode *Steinernema carpocapsae* (Platzer et al., 1999).

In the femoral muscle of locust, AK and arginine phosphate function as a temporal energy buffer (Schneider et al., 1989). Arginine phosphate concentrations were four times higher than ATP in the femoral muscle of locust at rest. It was proposed that when the locust jumps the ATP levels are maintained by using the phosphoarginine pool. In contrast, the AK-arginine phosphate system in locust did not appear to be involved in buffering energy levels of locust flight muscle during the first 3 seconds of flight because ATP levels fell while the phosphoarginine pool was not exhausted by the end of 3 seconds of flight. Nevertheless, in addition to locust, AK has been localized to muscle fibers of *D. melanogaster* (Lang et al., 1980) and lobster *Homarus vulgaris* (Benyamin and Robin, 1976). These data imply a function for AK in muscle movement. In the parasitic nematode *S. carpocapsae,* phosphoarginine may also function as an energy source mobilized during the transition between anaerobic and aerobic metabolism that may often occur in water-saturated soils (Platzer et al., 1999). Moreover, functional expression of AK in transgenic yeast (*Saccharomyces cerevisiae*), which does not normally contain a phosphagen kinase system, conferred resistance to transient stresses in the yeast by buffering the ATP pool (Canonaco et al., 2002). These results suggest that, in addition to a role in muscle contraction during periods of high activity, AKs also may have an important role in providing a source of ATP during short intervals of stress.

The soybean cyst nematode (SCN), *Heterodera glycines,* is the major pest of soybean and is responsible for an estimated loss of $700 million of soybean in the United States each year (Wrather et al., 2001). The second-stage juveniles (J2) are motile from egg hatch until establishment of a syncytium in the soybean root. The females are then sessile for the rest of their life cycle whereas the males subsequently become motile to fertilize the females. Here we describe the sequence for two SCN AKs (Hg-AK1 and Hg-AK2) and their expression patterns throughout the SCN life cycle.

Materials and Methods

cDNA sequence and analysis: The SCN clones ro62b05 and ro62b12 (accession numbers BI396813 and BI396819) were obtained from Washington University

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Genome Sequencing Center, St. Louis, Missouri (from library OP25), amplified in *Escherichia coli* and plasmid isolated using the QIAprep miniprep system (Qiagen, Santa Clarita, CA). The cDNA inserts were sequenced using the ABI Big Dye Terminator Cycle Sequencing Kit and run on the ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Applied Biosystems, Foster City, CA). Both clones were completely sequenced in both directions and found to be identical except for two nt at the 3′ end (Fig. 1). However, both cDNA clones were truncated at the 5′ ends. To obtain the 5′ sequence, nested primers were designed (Fig. 1). For the first round of PCR, a 3′ gene-specific primer was used in a linear reaction using 1 µl of an SCN J2 cDNA library, a directional phage library (OP50) from E. L. Davis (North Carolina State University), in a 50-µl volume for 35 cycles. For the second round of PCR, 1 µl from the first reaction was used as template, and a nested 3′ gene-specific primer plus a T3 vector primer were used for 35 additional cycles. The PCR product was gel-purified and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). The 5′ clone was sequenced and assembled with sequences for clones BI396813 and BI396819 using Lasergene software (DNASTAR Inc., Madison, WI).

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   1 ACCAAAATGGCTGTCCCTCAGGAAGTAATTGACAAAATTGAGGCTGGCTACAAAAAGCTT
         M  A  V  P  Q  E  V  I  D  K  I  E  A  G  Y  K  K  L

  61 CAGGCGGCGGCTGATTGCAAATCGTTGTTGAAGAAGTACCTCACCAAGGACGTCGTCGAT
     Q  A  A  A  D  C  K  S  L  L  K  K  Y  L  T  K  D  V  V  D

 121 GCATGCAAGGACAAGCGTACCAAGCTCGGAGCGACTTTCTTGGACGTCATCCAGTCGGGG
     A  C  K  D  K  R  T  K  L  G  A  T  F  L  D  V  I  Q  S  G

 181 GTTGCCAACTTGGACAGTGGTGTCGGCGTGTATGCGCCAGATGCCGAGGCTTACACACTC
     V  A  N  L  D  S  G  V  G  V  Y  A  P  D  A  E  A  Y  T  L

 241 TTCAAACCATTGTTCGACCCGATCATCAACAACTATCATGGTGGCTTTGGACCGAGCAGC
     F  K  P  L  F  D  P  I  I  N  N  Y  H  G  G  F  G  P  S  S

 301 AAGCAACCGGCAACGGACCTTGGTGAGGGGAAGACTCAACTGCCGACTGACTTGGACCCG
     K  Q  P  A  T  D  L  G  E  G  K  T  Q  L  P  T  D  L  D  P

 361 GAAGGGAAATTCATCAATTCGACTCGCGTTCGCTGCGGCCGCTCTCTTCAGGGGTATCCA
     E  G  K  F  I  N  S  T  R  V  R  C  G  R  S  L  Q  G  Y  P
                                                  <--Primer 2--
 421 TTCAACCCATGCTTGACCAAGGAGAATTACGTCGAGATGGAATCAAAGGTCAAAGCGGTC
     F  N  P  C  L  T  K  E  N  Y  V  E  M  E  S  K  V  K  A  V
            <-------Primer 1--------
 481 TTCGAGCAGCTGAAGGGCGATGCCGAGTTGGGAGGCACCTACTATCCGCTCCAGGGCATG
     F  E  Q  L  K  G  D  A  E  L  G  G  T  Y  Y  P  L  Q  G  M

 541 ACCAAGGACGTCCAAAATCAGCTGATTGCCGACCACTTCTTGTTTAAAGAGGGCGACCGT
     T  K  D  V  Q  N  Q  L  I  A  D  H  F  L  F  K  E  G  D  R

 601 TTTCTGCAGGAGGCCAATGCTTGCCGTTATTGGCCCACCGGACGCGGAATTTCCATAAC
     F  L  Q  E  A  N  A  C  R  Y  W  P  T  G  R  G  I  F  H  N

 661 GCCAAGAAAACCTTTTTGGTGTGGGTCAACGAGGAAGATCACCTCCGTATCATTTCGATG
     A  K  K  T  F  L  V  W  V  N  E  E  D  H  L  R  I  I  S  M

 721 CAAAATGGCGGCAATGTTGGACAAGTGCTGGAGCGTCTCATAAAGGGAGTCAAGGCCATC
     Q  N  G  G  N  V  G  Q  V  L  E  R  L  I  K  G  V  K  A  I

 781 GAATCGAAAGTTCCGTTTTCGCGTGACGACCGTCTTGGCTGGCTGACTTTCTGTCCTACC
     E  S  K  V  P  F  S  R  D  D  R  L  G  W  L  T  F  C  P  T

 841 AACTTGGGAACGACTGTTCGCGCGAGTGTGCATATCCGACTTCCGAAGATCAGCGCAAAA
     N  L  G  T  T  V  R  A  S  V  H  I  R  L  P  K  I  S  A  K

 901 CCCGACTTCAAGTCCATCTGCGACGGCCTGAAATTGCAAATCCGCGGGATTCACGGCGAA
     P  D  F  K  S  I  C  D  G  L  K  L  Q  I  R  G  I  H  G  E

 961 CATTCCGAGAGCGCTGGCGGCGTTTACGACATCAGCAACAAGGCCCGTCTTGGTCTCACG
     H  S  E  S  A  G  G  V  Y  D  I  S  N  K  A  R  L  G  L  T

1021 GAGTTCGAAGCGGTGAAGCAGATGTACGACGGAGTGAAACACCTGATCGAATTGGAGAAG
     E  F  E  A  V  K  Q  M  Y  D  G  V  K  H  L  I  E  L  E  K

1081 AAAGCATAATGGGAGGACCGATCTATGATGATGACTTTATATTGCGTAATGTGGAGATTT
     K  A  *
                                C
1141 GCTAGTTGTTCTGAATCTCTTAGGCTTTAACTATTTAATTATTGTCCCAAAATCGTGCTT
                                T
1201 CATCCGTATGATTATTTCCGTGCATAAATTTGTTCGTGTTCAAAAAAAAAAAAAAAA
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FIG. 1. Nucleotide sequence and translation of the *Heterodera glycines* AK 1, Hg-AK1 (accession number AY191835). The N-terminal and C-terminal ATP-guanido phosphotransferase domains are underlined in the amino acid sequence. Two separate clones (BI396813 and BI396819) from an *H. glycines* library (OP25, Washington University Genome Sequencing Center, Saint Louis, MO) were fully sequenced obtaining both forward and reverse sequence for each clone. The 5′ nucleotide sequence from position 1 to 411, which is not found in BI396813, BI396819, or the Hg-AK1 contig, was obtained from a cDNA in a different *H. glycines* library (OP50) from E. L. Davis (North Carolina State University). The nucleotide sequences used to prepare nested 3′ to 5′ PCR primers to clone the 5′ cDNA are indicated as Primer 1 and Primer 2 with an arrow above the nucleotide sequence. The boldface characters in the 3′ end with one character above the other are the only differences between the two sequences for BI396813 (above) and BI396819 (below). The bottom sequence is the more common sequence found in the EST sequences in the contig for Hg-AK1.

To obtain the full-length sequence for the other AK transcript, Hg-AK2, PCR primers were prepared from the consensus sequence for the alignment of four SCN ESTs and used in combination with the appropriate T3 or T7 vector primer to amplify 3′ and 5′ ends of homologous sequences in the directional OP50 SCN cDNA library. Two PCR fragments (3′ and 5′) were obtained and gel-purified. Each of the two PCR fragments possessed 418 nt of overlapping sequence. The two purified overlapping fragments were joined by PCR with the T3 and T7 vector primers to generate a single full-length clone, Hg-AK2 (Fig. 2). Multiple PCR fragments and full-length clones were partially sequenced and a full-length clone fully sequenced in both directions as previously described.

Generation of contiguous sequences (contigs) and phylogenetic analysis: The SCN expressed sequence tag (EST) database was searched for sequences annotated as having similarity with AKs. Contigs were generated from this data set using the Genetics Computer Group (GCG) Fragment Assembly suite of programs (Accelrys, San Diego, CA). After this initial alignment, the consensus sequence for each contig and singleton was used to again search the SCN EST database for more ESTs that were not annotated as having similarity with AKs. The resulting consensus sequence for each contig and singleton was then used to perform a FastX (GCG) search of the Swisspro database to confirm high similarity with AKs (Table 1). A similar approach was used to create AK contigs for ESTs from several other nematode species. The consensus sequences for nematode contigs that contained nearly full-length open reading frames were translated into peptide sequences and a dendrogram generated with other AK proteins from the Swisspro database. The GCG programs Pileup, Distance and Growtree were used to prepare the dendrogram using the Kimura algorithm specifically designed for analysis of protein sequences.

Nematode material and plant inoculation: The SCN population NL1-RH, which interacts with soybean differentials in the manner of race 3, has been maintained on greenhouse-grown soybeans at the U.S. Department of Agriculture, Agricultural Research Service (USDA, ARS) in Beltsville, Maryland, for many years and can be obtained from the SCN Stock Center (T. L. Niblack,

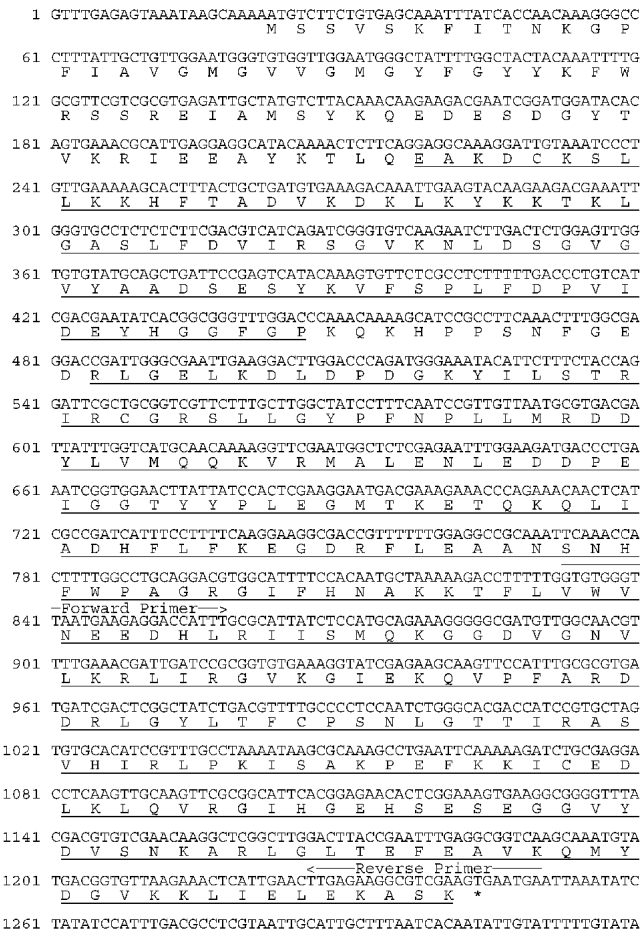

FIG. 2. Nucleotide sequence and translation of the *H. glycines* AK 2, Hg-AK2 (accession number AY225522). The N-terminal and C-terminal ATP-guanido phosphotransferase domains are underlined in the amino acid sequence. The nucleotide sequences used to prepare forward and reverse primers for PCR cloning are indicated with an arrow above the corresponding sequences. The Hg-AK2 PCR fragments were obtained by amplification of clones in the *H. glycines* cDNA library OP50 (E. L. Davis, North Carolina State University).

University of Illinois, Champaign-Urbana, IL). For these experiments, the nematodes were propagated on *Glycine max* cv. Williams 82 plants for 3 months prior to harvesting cysts. Cysts were isolated from root debris as previously described (Krusberg et al., 1994). Cysts were further purified by centrifugal flotation in a 35% to 40% sucrose solution (w/v) and eggs hatched at 25 °C for 2 days (Barker, 1985; Jenkins, 1964). Unhatched eggs and J2 were separated from each other by filtering through a 41-µm nylon mesh. A fraction of the eggs and J2 were frozen in liquid nitrogen for subsequent RNA extraction. The remaining J2 were then concentrated by centrifugation to 5,000 nematodes/ml.

Glycine max cv. Williams 82 seeds were germinated and grown in Perlite (Geiger, Harleysville, PA) in the greenhouse. Two-week-old seedlings were washed free of Perlite and the roots placed between moist paper towels. The seedlings were combined into groups of five seedlings per treatment and inoculated by pipeting

TABLE 1. Percent identity among the open reading frames in full-length sequences in *H. glycines* and *C. elegans* and the EST singletons Hg-AK4 and Hg-AK5. Percents above diagonal indicate nucleotide identity; those below the diagonal indicate amino acid identity. Translations of Hg-AK4 and Hg-AK5 in all three forward reading frames were interrupted by multiple stop codons and, therefore, were not included in peptide comparisons. Percent identities were calculated using the GCG gap algorithm (Accelrys). The exponents (E values) in the header indicate the probability score for the expect value of each AK with the protein sequence for *C. elegans* AK1 (CeAK1) in a FastX search of the Swisspro database (GCG, Accelrys).

Sequence name	Hg-AK1 10^{-134} (full)*	Hg-AK2 10^{-119} (full)	Hg-AK4 10^{-13} (mid cds)	Hg-AK5 10^{-21} (mid cds)	CeAK1 10^{-152} (full)	CeAK2 10^{-119} (full)
Hg-AK1	100	66	73	73	73	65
Hg-AK2	71	100	54	55	63	61
Hg-AK4	—	—	100	62	64	60
Hg-AK5	—	—	—	100	57	55
CeAK1	82	67	—	—	100	69
CeAK2	71	66	—	—	71	100

* full, full-length coding sequence; mid cds, coding sequence in middle of protein.

2,500 (0.5 ml) J2/seedling. The roots were then sprinkled with moist Perlite and covered with a moist paper towel. The groups of five seedlings each were kept in a moist environment for 1, 2, 4, 8, 12, 20, and 30 days in a growth chamber with 15 hours of light per 24-hour cycle. After the desired incubation interval, one of five seedlings in each group was collected for fuchsin staining (Byrd et al., 1983) to monitor nematode development and approximate number of infections.

Reverse Transcriptase PCR: RNA was extracted from roots as previously described (Koehler et al., 1996). RNA from eggs and J2 was extracted using an RNeasy plant mini kit from Qiagen (Santa Clarita, CA). RNA from eggs and juvenile nematodes was diluted 1:100 with uninoculated soybean root RNA. First-strand cDNA synthesis was completed with oligo dT and 10 µg of each RNA sample in a 60-µl reaction volume using the Superscript First-strand Synthesis System for reverse transcriptase polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA). Subsequent PCR was completed for each primer pair using 1/20 volume of the first-strand synthesis and the Platinum *Taq* PCR system (Invitrogen, Carlsbad, CA). Polymerase chain reaction was started with 94 °C for 2 minutes followed by 25, 30, or 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 70 °C for 60 seconds. Polymerase chain reaction was finished with 70 °C for 10 minutes ending with a 4 °C hold. The PCR products were separated on a 1.5% TAE (Tris, Acidic acid, EDTA) agarose gel, imaged with a video camera, and quantified with the NIH Image software version 1.62 (available at http://rsb.info.nih.gov/nih-image/download.html).

RESULTS

Characterization of two AK cDNAs: Initially, partial 5′ end sequences for two SCN clones ro62b05 and

ro62b12 (accession numbers BI396813 and BI396819, respectively) were identified in the SCN EST database as having similarity with an AK in *Caenorhabditis elegans.* Both clones were sequenced to obtain full-length sequence for both clones (Fig. 1). The sequences for these clones were identical except for two nucleotides (Fig. 1). Alignment of these sequences with that of the *C. elegans* AK sequences indicated that both clones were missing much of the 5′ ends of the transcripts. A subsequent BLASTX search with the SCN AKs ro62b05 and ro62b12 and two *C. elegans* AKs identified 251 sequences in the public *H. glycines* EST database that had similarity with these AKs (Table 1). Surprisingly, these 251 ESTs represent more than 5.8% of the 4327 SCN ESTs in the database. Alignment of all 251 sequences produced two contigs and three singletons. Interestingly, 244 of the 251 ESTs grouped into a single contig (Hg-AK1) that included both ro62b05 and ro62b12. Although there is an abundant number of ESTs in the Hg-AK1 contig, comparison of the consensus sequence for this contig with the complete DNA sequences of AKs from *D. melanogaster* and *C. elegans* suggested that the SCN clones used for EST sequencing were truncated and did not contain the 5′ portion of the open reading frame. To obtain 5′ sequence for Hg-AK1 transcript, nested PCR primers were designed (Fig. 1) to specifically amplify the 5′ portion of longer clones in a phage cDNA library provided by E. L. Davis (North Carolina State University). The assembly of a near full-length cDNA sequence for Hg-AK1 contains a large open reading frame of 1080 nt encoding an inferred 360 amino acid (aa) sequence and a 133 nucleotide (nt) 3′ untranslated region ending with a polyadenylate tail (Fig. 1). Alignment of the derived protein sequence for Hg-AK1 with other AKs indicates that the first methionine in the sequence (Fig. 1) is likely to be the native start of translation. The resulting protein has a molecular weight of 40 kDa and a theoretical isoelectric point of 8.33.

In addition to Hg-AK1, alignment of AK EST sequences produced another contig, Hg-AK2, and three singletons. The Hg-AK2 contig was made up of four sequences encoding only the 3′ end of a putative AK. A full-length clone for Hg-AK2 was obtained by PCR amplification of homologous clones in a directional phage SCN cDNA library (OP50) and sequenced (Fig. 2). The 3′ sequence for the resulting PCR clone had 100% identity with the 538 nt Hg-AK2 contig. Moreover, the 5′ portion of the Hg-AK2 sequence had 100% identity with the first 300 nt of one of the three singleton ESTs (Hg-AK3, accession number BI749633). After the first 300 nt of this EST, the Hg-AK2 sequence had progressively more mismatches as the EST sequence reached its end. The full-length Hg-AK2 sequence contains an open reading frame of 1221 nt encoding a 407 aa peptide with a predicted molecular weight of 46 kDa and isoelectric point of 9.58.

Two additional EST sequences, Hg-AK4 and Hg-AK5 (accession numbers BI749659 and BI748461, respectively), were identified in the SCN database that had high sequence identity to Hg-AK1 and *C. elegans* AK1 (Table 1) but didn't align to form a contig with any other sequence in the SCN EST database. Less stringent alignments of Hg-AK4 and Hg-AK5 with other AKs displayed sequence mismatches that were evenly distributed throughout the length of the alignments. The even distribution of mismatches indicated that these ESTs were not simply poor sequence data but more likely represented sequences for different AK genes. The PCR primer pairs were prepared for each of the Hg-AK contigs and singletons. Primers for Hg-AK4 and Hg-AK5 did not, however, produce a discernable RT-PCR product of the expected size. All of the Hg-AK primer sets were then tested with SCN genomic DNA. Again, Hg-AKs 4 and 5 did not produce a prominent PCR product whereas the others did.

Comparison of the SCN AKs with other AKs: Percent nucleotide and amino acid identities between the SCN and *C. elegans* AK sequences are shown in Table 1. In addition to SCN ESTs, ESTs having similarity with AKs in other nematode species were also aligned to create 20 contigs and 14 singletons in 16 different nematode species. Alignment and phylogenetic analysis of near full-length protein sequences for the nematode contigs (excluding all singletons and short contigs) and protein sequences for other AKs identified in the Swisspro database are displayed as a dendrogram (Fig. 3). In this dendrogram only the sea anemone AK includes two domains for each of the N- and C-terminal phosphotransferase domains (Suzuki et al., 1997b). The sea anemone protein is twice as large as all the other AKs included in the dendrogram.

Expression patterns for SCN AKs: The PCR primer pairs were prepared for each of the Hg-AK contigs and singletons. In addition, primers were prepared for the SCN elongation factor 1β (Hg-EF1b), an SCN sequence having similarity with a heat shock protein (Hg-Hsp1), an SCN β-1,4-endo glucanase (Hg-Eng-1), and a soybean 28S ribosomal RNA (Gm-rRNA) (Table 2). As mentioned previously, primers for Hg-AK4 and Hg-AK5 did not produce a discernable RT-PCR product of the expected size and therefore were not included in the following temporal expression assays. Reverse transcriptase polymerase chain reaction was performed for all stages of nematode development (Fig. 4). The amount of RNA from SCN eggs and J2 used for RT-PCR was adjusted by diluting with uninoculated soybean root RNA to a ratio of 1:100. This was empirically determined to be the best dilution to produce approximately equivalent amounts of PCR product for the Hg-EF1b PCR product. After first strand synthesis, the PCR amplification was completed with either 25, 30, or 35 cycles. A greater number of cycles was used for the less

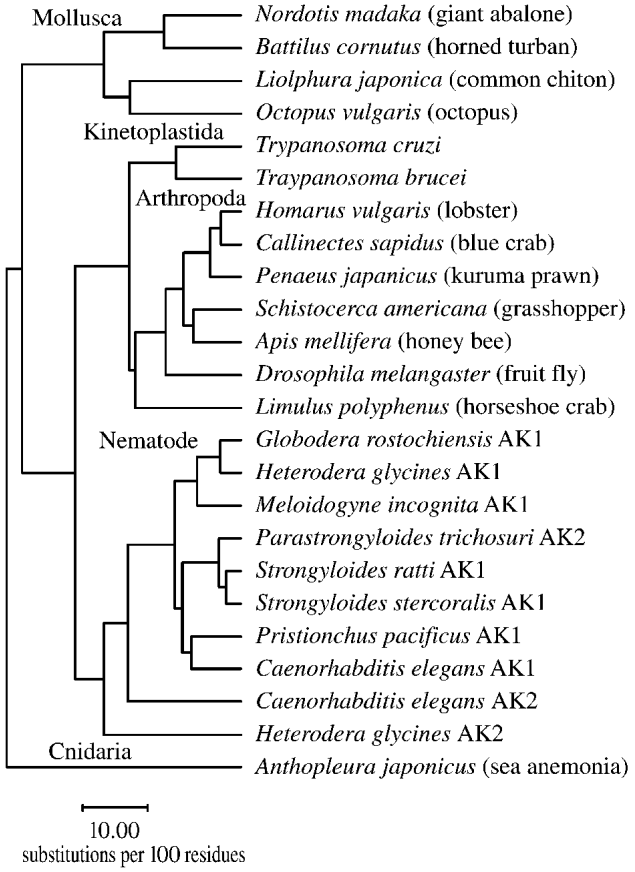

FIG. 3. Dendrogram for phylogenetic analysis of AK peptides in the Swisspro database and the open reading frame translations for nematode AKs with near full-length consensus sequences. The accession number for a representative EST in each nematode contig and the number of ESTs per contig in parentheses are: *C. elegans* AK1, au108959 (230); *C. elegans* AK2, au199777 (31); *G. rostochiensis* AK1, aw506333 (20); *H. glycines* AK1, ay191835 (244); *H. glycines* AK2, ay225522 (5); *M. incognita* AK1, aw828855 (33); *P. trichosuri* AK2, bi863725 (6); *P. pacificus* AK1, aa193928 (11); *S. ratti* AK1, bi073820 (9); and *S. stercoralis* AK1, be580187 (22).

abundant transcripts to achieve easily visible and quantifiable bands on a gel.

The bar graph underneath each image indicates the relative quantity of each PCR product within that grouping of reactions normalized to the quantity of the Hg-EF1b PCR product. The Gm-rRNA bar graph was not normalized to any other PCR product and is used to indicate equivalent amplification among the different RNA preparations. Hg-AK1 and Hg-AK2 have very similar expression patterns and, based on this semiquantitative RT-PCR, are of similar abundance (Fig. 4).

The elongation factor (Hg-EF1b), heat-shock-1 (Hg-Hsp1), and endoglucanase (Hg-Eng-1) were added as controls for comparison to the AKs. Hg-EF1b is fairly constitutive, increasing as the nematode grows and expands with age. However, the increase in Hg-EF1b as the nematodes grow is partially offset by a decreasing number of nematodes that successfully form feeding structures and develop into adults at later days post inoculation (dpi). The numbers of nematodes counted inside a sample seedling root were: 415 nematodes at 1 dpi, 422 at 2 dpi, 216 at 4 dpi, 64 at 8 dpi, and 91 at 12 dpi. Many more nematodes penetrated the roots at 1 and 2 dpi than formed successful feeding structures and developed into swollen females at later dpi. Nematodes at 20 dpi and 30 dpi were not counted because the swollen females often broke free of the roots and washed away. At 30 dpi some eggs had already hatched to release a new population of juvenile nematodes that reinfected the roots. The Hg-Eng-1 transcript decreases as the nematode develops in the root, whereas HgHsp1 transcript increases during development and then almost disappears in eggs and J2 (Fig. 4).

DISCUSSION

Although AKs are fairly abundant in the nematode EST databases, which included 154,576 sequences for 23 different nematode species, characterization of a multigene family for AKs and the expression patterns for parasitic plant nematodes have not been previously reported. In particular, the SCN EST database includes a disproportionately large number of AK ESTs. To determine the expression patterns for the Hg-AK genes during the entire SCN life cycle, we chose to use a semiquantitative RT-PCR approach to enhance the detection of relatively low abundant AK transcripts in a mixture of plant and nematode RNA (approximately 100:1 ratio of plant to nematode RNA). To ensure that our RT-PCR gave reliable expression pattern data, several controls were incorporated (Fig. 4). In addition to a constitutive expression pattern for the SCN elongation factor (Hg-EF1b) (Ursin et al., 1991), the expression pattern exhibited for Hg-Eng-1 is consistent with earlier reports that demonstrate that this endo-1,4-glucanase is most abundant in J2 as they migrate through the plant tissue toward the vascular bundle (de Boer et al., 2002). Also consistent with earlier reports is the expression pattern for Hg-Hsp1, whose transcript increases only after the feeding structure has been initiated (de Boer et al., 2002).

The PCR primer pairs were successfully prepared for Hg-AK1 and Hg-AK2. Both Hg-AK1 and Hg-AK2 are expressed constitutively and at an approximately equal concentration in the nematode. The equal concentration result is in contrast to the very large number of ESTs for Hg-AK1 as compared to the very few ESTs for Hg-AK2. It is unclear why Hg-AK1 represents more than 5% of the ESTs in the SCN database. No other AK for any of the other species of nematodes is represented by such a high percentage of the ESTs in the database for that species. However, it's possible that the nematodes used to prepare the OP25 library that was then used for EST sequencing may have been exposed to an environmental signal (e.g., anaerobic stress) that greatly enhanced the expression of the Hg-AK1 transcript prior to isolation of RNA.

TABLE 2. Primers used for RT-PCR. Mismatches between the Hg-AK1 and Hg-AK2 primers and the full-length sequence for Hg-AK2 and Hg-AK1, respectively, are indicated as a lowercase letter. The Genbank accession numbers for the sequences used for primer design are: Hg-AK1, AY191835; Hg-AK2, AY225522; Hg-EF1b, BF014332; HG-Hsp1, AF273728; Hg-Eng-1, AF006052; Gm-rRNA, L36615.

Sequence name	5′ forward primer	3′ reverse primer	PCR length
Hg-AK1	AcgTcGagATGgAAtcAAAGGTcaaAg	aGGaCAgAAaGTCAGccAGCCaAG	389
Hg-AK2	GTGTGGGTtAAtGAaGAgGAcCAttT	TCATtcaCtTcGAcgccTtCTCaa	418
Hg-EF1b	TCAACAATAAGCTCACATCGGGAC	GCCTTTTTCTCCGCATAAGCCTTC	390
Hg-Hsp1	AATTGTCACCGCTGGACTGCT	GCATCCATTCTTTCTTCCTCCGTT	317
Hg-Eng-1	CAGCATCTCTGTAGTCCCATCCAA	TCCCATTCTTTTCCTCCCATTTTGC	300
Gm-rRNA	CAATCGGGCGGTAAATTCCGAC	TTCCCTCCCGACAATTTCAAGC	130

Phosphoarginine is an important energy source to replenish the ATP pool during periods of intense activity such as insect flight (Lang et al., 1980; Newsholme et al., 1978) or during short periods of stress (Alonso et al., 2001; Canonaco et al., 2002; Platzer et al., 1999).

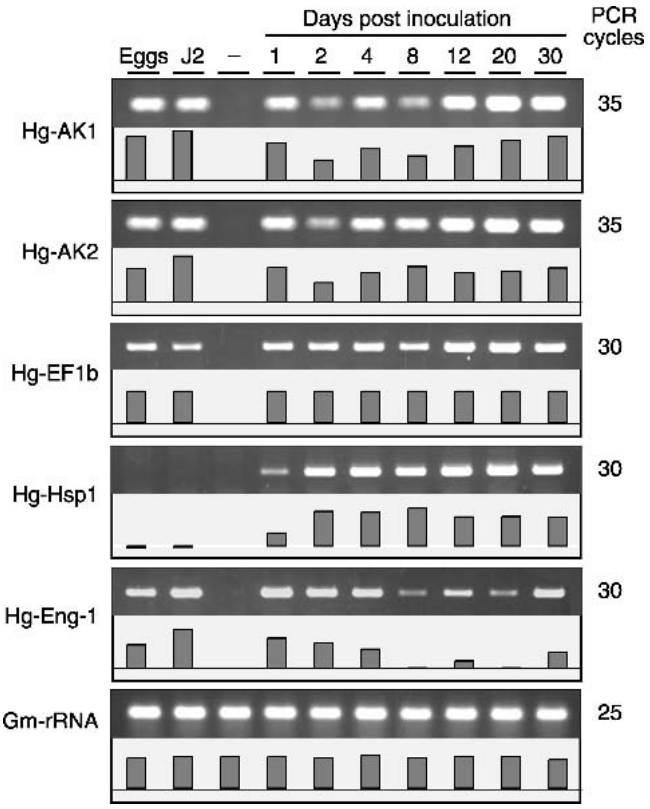

FIG. 4. RT-PCR of transcripts for two SCN AKs (Hg-AK1 and Hg-AK2), an SCN elongation factor 1-b (Hg-EF1b), an SCN heat-shock-like protein (Hg-Hsp1), an SCN β-1,4-endo glucanase (Hg-Eng-1), and a soybean 18S ribosomal RNA (Gm-rRNA). In the first two lanes (eggs and J2), RNA from 2-week-old uninoculated soybean roots (–) was spiked with 1/100 (wt/wt) of SCN RNA isolated from eggs or newly hatched J2 nematodes, respectively. In lanes labeled as days post inoculation 2-week-old soybean roots were inoculated with juvenile J2 nematodes and kept in a moist environment for 1, 2, 4, 8, 12, 20, and 30 days at the end of which the roots were harvested for RNA extraction. The bar graph underneath each image indicates the relative quantity of each PCR product within that grouping of reactions normalized to the quantity of the Hg-EF1b PCR product in that RNA preparation. The Gm-rRNA bar graph was not normalized to any other PCR product and is used to indicate equivalent amplification among the different RNA preparations.

When yeast (*S. cerevisiae*) was transformed with an AK, the transgenic yeast demonstrated greater resistance to starvation stress and transient changes in pH (Canonaco et al., 2002). Similarly, it was concluded that AK was important to the survival of *Trypanosoma cruzi* when the protozoa was placed under starvation conditions (Alonso et al., 2001). Moreover, AK activity increased markedly in the pathogenic insect nematode *S. carpocapsae* when the nematode was transferred from aerobic to anaerobic conditions (Platzer et al., 1999). Assuming that stress conditions may occur abruptly and unpredictably during the nematode life cycle, it might be expected that AK would need to be expressed constitutively at a low level throughout nematode development. Indeed, the expression pattern for Hg-AK1 and Hg-AK2 in SCN indicates that these genes are constitutively expressed during SCN development, including eggs (Fig. 4). Nevertheless, environmental cues like anaerobic stress may greatly increase AK expression above the levels observed under our conditions.

In the CK phosphagen system in vertebrates, a mitochondrial CK isozyme synthesizes phosphocreatine and a cytoplasmic isozyme breaks it down to generate ATP (Saks et al., 1994). However, the AK phosphagen system in *D. melanogaster* appears to be different. Arginine kinase in *D. melanogaster* is encoded by a single gene that functions in both the forward and reverse reactions in the mitochondria and cytoplasm, respectively (Munneke and Collier, 1988). In SCN, because there are multiple genes for AKs, separate isoenzymes in the mitochondria and cytoplasm can't be ruled out (Table 1). Sequence comparison of the sequences for Hg-AK1, Hg-AK2, and the two EST singletons indicates considerable divergence between the different isoforms of AK in SCN (Table 1), which supports a model of functional divergence as well. However, redundancy of AKs in nematodes simply may reflect its essential role in the nematode life cycle or a specialized expression pattern of one or more genes in cells having a particularly high fluctuation in metabolic rates akin to that in the muscles of insects. More complete sequencing of putative transcripts for Hg-AK4 and Hg-AK5 and immunological approaches combined with in situ hybridization with antibody and cDNA probes, respectively, may dis-

tinguish among these possibilities. Nonetheless, the abundance of AK sequences in the SCN EST database and the relatively strong signal in RT-PCR (Fig. 4) suggest that the AK phosphagen system is very important for nematode survival and possibly a good target for control of parasitic nematodes through expression of an RNA interference (RNAi) construct in the host (Urwin et al., 2002). Furthermore, AKs are not present in vertebrate systems, making it even more attractive as a target in agricultural products that are consumed by humans and livestock.

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