A Chorismate Mutase from the Soybean Cyst Nematode *Heterodera glycines* Shows Polymorphisms that Correlate with Virulence

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Parasitism genes from phytoparasitic nematodes are thought to be essential for nematode invasion of the host plant, to help the nematode establish feeding sites, and to aid nematodes in the suppression of host plant defenses. One gene that may play several roles in nematode parasitism is chorismate mutase (CM). This secreted enzyme is produced in the nematode's esophageal glands and appears to function within the plant cell to manipulate the plant's shikimate pathway, which controls plant cell growth, development, structure, and pathogen defense. Using degenerate polymerase chain reaction primers, we amplified and cloned a chorismate mutase (*Hg-cm-1*) from *Heterodera glycines*, the soybean cyst nematode (SCN), and showed it had CM activity. RNA in situ hybridization of *Hg-cm-1* cDNA to SCN sections confirms that it is specifically expressed in the nematodes' esophageal glands. DNA gel blots of genomic DNA isolated from SCN inbred lines that have differing virulence on SCN resistant soybean show *Hg-cm-1* is a member of a polymorphic gene family. Some *Hg-cm* family members predominate in SCN inbred lines that are virulent on certain SCN resistant soybean cultivars. The same polymorphisms and correlation with virulence are seen in the *Hg-cm-1* expressed in the SCN second-stage juveniles. Based on the enzymatic activity of *Hg-cm-1* and the observation that different forms of the mutase are expressed in virulent nematodes, we hypothesize that the *Hg-cm-1* is a virulence gene, some forms of which allow SCN to parasitize certain resistant soybean plants.

Additional keywords: parasitism gene, salicylic acid, phytoalexin.

In order for plant parasitic nematodes to infect plants, they must produce an assortment of parasitism genes. These genes may be expressed in nematode organs that produce externally secreted proteins, such as the esophageal glands, amphids, or hypodermis, with the esophageal gland parasitism genes being the most intensely studied to date (Davis et al. 2000). To penetrate and migrate through plant tissue, nematodes produce and secrete cellulases (Rosso et al. 1999; Smant et al. 1998) and pectate lyases (De Boer et al. 2002; Doyle and Lambert 2002; Popeijus et al. 2000b) from their esophageal gland cells. Once in the root, sedentary endoparasitic nematodes, such as the root-knot and cyst nematodes, are known to express a variety

of parasitism genes in their esophageal glands (Davis et al. 2000; Gao et al. 2001; Qin et al. 2000; Wang et al. 2001). Most of these parasitism genes have no known function but are thought to be involved in establishment of nematode feeding cells. The suppression of host plant resistance to assist the nematode in establishing a permanent feeding association is another process nematode parasitism genes are thought to control (Davis et al. 2000). While little is known about which parasitism genes control this process, some progress has been made in this field in recent years. A putative avirulence gene expressed in the amphids of the root-knot nematode has been isolated, but its biochemical mode of action is unclear (Semblat et al. 2001). The genetics of nematode virulence has been examined in cyst nematodes, but the gene or genes responsible for virulence have not been cloned and characterized (Dong and Opperman 1997; Dong et al. 1997; Janssen et al. 1991; Triantaphyllou 1975).

Out of all the parasitism genes cloned, only a nematode chorismate mutase (CM; EC 5.4.99.5) has an enzymatic activity that could assist the nematode in parasitizing resistant plants (Lambert et al. 1999). Chorismate mutase is an enzyme in the shikimate pathway, a primary metabolic route found in plants, bacteria, fungi, and protozoans (Roberts et al. 1998; Schmid and Amrhein 1995). The shikimate pathway does not exist in animals, but a secreted form of CM is present in the root-knot nematode (Doyle and Lambert 2003; Lambert et al. 1999) and the potato cyst nematode (Jones et al. 2002; Popeijus et al. 2000a). Because plants have the shikimate pathway and nematodes have a secreted form of CM, this nematode enzyme is thought to be involved in the alteration of the plant's pathway. The plant shikimate pathway is an important metabolic route that produces the aromatic amino acids, plant hormones, cell wall components, and numerous secondary metabolites that have plant defense activity (Dewick 1998). Due to the multifunctional nature of the shikimate pathway, nematode CMs could change any or all of these important plant processes.

We have cloned a cDNA from the soybean cyst nematode (SCN) that encodes a chorismate mutase. This new nematode CM was characterized using DNA gel blotting, in situ hybridization, and an enzymatic assay. Our characterization also involved the use of SCN inbred lines that have differing virulence on SCN resistant soybeans to assess whether the SCN CM has any correlation to SCN virulence.

RESULTS

Cloning and sequence analysis of *Hg-cm-1*.

Poly A+ RNA was isolated from SCN second-stage juveniles that had been in the soybean root four days or less, and

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Nucleotide and amino acid sequence data are available from the GenBank database under accession numbers AY160225 for the *Hg-cm-1A* cDNA, AY227803 for the *Hg-cm-1B* cDNA, and AY160224 for the genomic clone.

then cDNA was synthesized and polymerase chain reaction (PCR)-amplified. Degenerate PCR primers were designed to hybridize to a sequence that is conserved between the *Meloidogyne javanica* chorismate mutase (*Mj-cm-1*) and that of *Globodera pallida* (*Gp-cm-1*) (Jones et al. 2002; Popeijus et al. 2000a). The degenerate PCR primer and a primer that hybridizes to the nematodes spliced leader sequence (SL-1) were used to amplify a 500-bp PCR product that contained the 5' end of the SCN CM cDNA sequence. The 3' end of the sequence was obtained using rapid amplification of cDNA ends PCR (Frohman 1990). Once assembled, the full *Heterodera glycines* chorismate mutase (*Hg-cm-1*) cDNA clone contained an 816-bp open reading frame encoding a 272-amino acid protein (AY160225). PCR primers that flank the open reading frame were used to PCR-amplify a genomic clone for *Hg-cm-1*. Once sequenced, the genomic clone of *Hg-cm-1* revealed a presence of three introns at 95 to 317, 500 to 593, and 813 to 873 bp (AY160224).

The predicted protein sequence of *Hg-CM-1* appears to have three regions or domains: an N-terminal domain, residues 1 to 84, with no homology to any known protein but containing a signal peptide (residues 1 to 23); a central domain, from residue 85 to 188 that has homology to bacterial and nematode chorismate mutase enzymes; and a C-terminal domain, residues 187 to 273, which has homology to other nematode chorismate mutases but has no homology to bacterial CMs (Fig. 1). Only the middle CM domain is predicted to have a function. On an amino acid level, the CM domain of *Hg-CM-1* is 70.4% identical to the *Globodera pallida* CM (*Gp-CM-1*), 30.6% identical to the *Meloidogyne javanica* CM (*Mj-CM-1*), 28.6%

identical to a *Erwinia herbicola* CM, and 16.5% identical to an *Escherichia coli* CM (Fig. 1). Out of the 11 active site residues found in the *E. coli* CM (Lee et al. 1995; Hur and Bruice 2002), 9 are conserved in *Hg-CM-1* or have similar residues. The only exceptions are for Arg 11, which is replaced with a Lys residue in cyst CMs, and Ser 84, which is replaced with an Asn residue. N-glycosylation sites are predicted to be at N43 and N98; these sites are also present in *Mj-CM-1*. The secondary structure predictions indicate that the CM domain of *Hg-CM-1* has the three alpha-helical sections typical of CM enzymes, indicating it could have CM activity (data not shown).

Hg-CM-1 enzymatic activity.

To test the CM activity of *Hg-CM-1*, we inserted the coding region minus the signal peptide into the bacterial expression vector pBAD/Thio-TOPO and transformed the CM-deficient *E. coli* JP2261 (Baldwin and Davidson 1981). Strain JP2261 has no functional CMs and, therefore, no bacterial CM activity that might mask that of *Hg-CM-1*. When JP2261 harboring *Hg-cm-1* cDNA was induced to produce *Hg-CM-1* protein by the addition of arabinose, robust CM activity was detected in bacterial cell extracts (Table 1). Since *Hg-CM-1* construct has six histidine residues at the C-terminus, we were able to partially purify the nematode CM. The dramatic increase in CM activity upon metal affinity chromatography indicates the CM activity purified with six histidine-tagged proteins and confirms *Hg-CM-1* enzymatic function (Table 1). Since *Hg-CM-1* contains an N-terminal domain not present in *Mj-CM-1*, we wanted to determine if this part of the protein was essential for CM activity. To address this issue, we constructed a shorter form of *Hg-cm-1* missing the N-

Fig. 1. Comparison of the protein domains of *Heterodera glycines* chorismate mutase (*Hg-CM-1A*) and *Meloidogyne javanica* chorismate mutase (*Mj-CM-1*). N = the N-terminal, CM = the chorismate mutase domain, and C = the C-terminal domain. The CM domain of *Hg-CM-1* is aligned with nematode and bacterial CMs. The numbers below the sequences indicate residues that contribute to the active site of the *E. coli* CM (ECCM), as determined by the X-ray crystal structure analysis (Lee et al. 1995), and the * marks the shifted arginine residue of *Mj-CM-1*. # marks the amino acid residues in the CM domain that are different between *Hg-CM-1A* and *Hg-CM-1B*. The changes in *Hg-CM-1B* chorismate mutase domain are: Q42 to N42, E50 to V50, and P102 to L102. N-glycosylation sites are predicted to be at N43 and N98. The species of nematode or bacterium, CM gene name, and accession number of the aligned sequences are, respectively: GPCM (*Globodera pallida*, *Gp-CM-1*, AJ487621), HGCM (*Heterodera glycines*, *Hg-CM-1A*, AY160225), MJCM (*Meloidogyne javanica*, *Mj-CM-1*, AF095949), EHCM (*Erwinia herbicola*, AroQ (P42517), and ECCM (*Escherichia coli*, PheA (P07023).

terminal domain and inserted this construct into the pBAD/Thio-TOPO vector. Once the construct was transformed into *E. coli* JP2261 cells and the shorter form of *Hg-CM-1* protein produced, we were able to assay for CM enzymatic activity. The shorter form of *Hg-CM-1* protein was fully functional, and the CM-specific activity increased when the *Hg-CM-1* protein was purified by metal affinity chromatography, indicating the N-terminal domain is not required for enzymatic function (Table 1). *Hg-CM-1* has no feedback activation or inhibition in the presence of aromatic amino acids, because the presence or absence of 0.5 mM of each amino acid did not significantly change the enzymatic activity (19.83 ± 0.27 nanomoles of prephenate formed per min per mg of protein, the standard deviation is shown).

Tissue localization of the nematode chorismate mutase.

To determine which nematode cells were expressing *Hg-cm-1* mRNA, we conducted RNA in situ hybridization experiments. Soybean roots were infected with SCN and were allowed to penetrate the roots for three days. The roots were fixed, embedded in paraffin, sectioned, and hybridized with digoxigenin-labeled *Hg-cm-1* cDNA antisense riboprobe. Hybridization signal was detected in subventral and dorsal gland cells (Fig. 2), as indicated by the blue hybridization signals. No signal was obtained from the negative control, the *Hg-cm-1* sense probe (data not shown).

Hg-cm-1 polymorphisms.

Since the *Hg-cm-1* cDNA has homology to bacterial chorismate mutases, it was therefore necessary to confirm that the cDNA originated from the nematode. DNA gel blots were conducted using *EcoRI*-digested SCN genomic DNA isolated from randomly selected SCN inbred lines and hybridized with radiolabeled *Hg-cm-1* cDNA. The resulting autoradiograph shows that *Hg-cm-1* cDNA hybridizes to between two and eight restriction fragments ranging in size from 2 kb to 12 kb and shows restriction fragment linked polymorphisms between SCN inbred lines (Fig. 3). The hybridization of *Hg-cm-1* to SCN genomic DNA and the lack of hybridization to soybean DNA support the nematode origin of the gene.

The hybridization of *Hg-cm-1* cDNA to the DNA gel blot produced a variable number of intense and faint hybridization signals within a SCN line. This hybridization variation could be due to alterations in the *Hg-cm* gene family member diversity, copy number, or sequence homology. While the hybridization pattern is complex with many variations between the SCN lines, two bands, *Hg-cm* "A" (2 kb) and "B" (2.2 kb), stand out clearly in all lines (Fig. 3). The varying hybridization intensities for the *Hg-cm* A and B bands within SCN inbred lines suggests *Hg-cm-1* copy number is variable within and between SCN inbred lines, since most lines have both forms to varying degrees (Fig. 3).

The SCN inbred lines used in the DNA gel blot differ from each other in their ability to parasitize different SCN-resistant

soybean varieties (Table 2). Interestingly, there is a correlation between the restriction fragment polymorphism and SCN virulence. The presence of a major B-band correlates with the ability of the nematode to reproduce on two of the SCN resistant soybean lines. SCN inbred lines TN15, TN19, TN20, and OP50 all have the B form of *Hg-cm-1*, and all grow well on resistant soybean PI90763 and PI89772, showing a high reproductive ability ranging from a female index of 49 to 88 (Fig. 3 and Table 2). The presence of a strong A band and a weaker or absent B band, however, correlates with a lower ability of the nematode to grow on PI90763 and PI89772, as is observed for inbred SCN lines TN1, TN2, TN3, TN16, and VL1 (Fig. 3 and Table 2).

To determine if the DNA gel blot polymorphisms were reflected in the expressed forms of *Hg-cm-1*, total RNA was extracted from SCN preparasitic second-stage juvenile inbred lines and cDNA-synthesized. The *Hg-cm-1* coding region was then PCR-amplified for SCN inbred lines containing A and B bands of *Hg-cm-1*. The cDNAs for SCN lines VI-1 (containing band A) and TN19 (containing band B) were cloned and sequenced. Seven nucleotide differences causing five amino acid changes were identified between the A and B mutases, which are now referred to as *Hg-cm-1A* and *Hg-cm-1B*. The changes in the CM domain of *Hg-CM-1A* are marked in Figure 1, additional changes in the C-terminal domain of *Hg-CM-1A* are I136 to K136 and E176 to K176. In *Hg-cm-1B*, one of the sequence changes caused the addition of an *RsaI* restriction site in the center of the coding region. We decided to use the *RsaI*

Fig. 2. RNA in situ localization of *Heterodera glycines* chorismate mutase (*Hg-cm-1*) mRNA in second-stage juvenile nematodes in soybean roots. All sections were hybridized with antisense digoxigenin-labeled riboprobe, followed by an alkaline phosphatase-conjugated antidigoxigenin antibody. Hybrids were detected by staining for alkaline phosphatase, which forms a dark blue to black precipitate. In all sections, the nematodes were in the roots four days or less. The expression pattern, indicated by the G arrows, shows *Hg-cm-1* is expressed in several esophageal gland basal cells. M = metacarpus, G = esophageal gland, and S = stylet.

Table 1. *Hg-CM-1* enzymatic activity^a

Plasmid	Protein type	CM activity
pBAD: <i>Hg-cm-L</i>	Cell extract	86
pBAD: <i>Hg-cm-S</i>	Cell extract	48
pBAD: <i>Hg-cm-L</i>	Purified	4988
pBAD: <i>Hg-cm-S</i>	Purified	636

^a Chorismate mutase (CM) activity was measured in protein extracts of *E. coli* JP2261 transformed with plasmid pBAD/Thio-TOPO expressing *Heterodera glycines* chorismate mutase (*Hg-CM-1*). The CM activities are expressed in nanomoles of prephenate formed per min per mg of protein. *Hg-CM-1* was purified using metal affinity chromatography. The plasmid pBAD: *Hg-cm-L* denotes the full-length form of *Hg-CM-1* minus the signal peptide sequence, and the pBAD: *Hg-cm-S* refers to a short form of *Hg-CM-1* with a deleted N-terminus of the protein.

restriction site to determine if the other SCN lines that had *Hg-cm-1B* genomic bands also expressed this form of the gene. To do this, we PCR-amplified *Hg-cm-1* cDNAs from eight different inbred lines, four with *Hg-cm-1A* and four with *Hg-cm-1B* and then subjected them to restriction enzyme digestion with *RsaI* to determine if this restriction polymorphism could discriminate between *Hg-cm-1A* and *Hg-cm-1B*. Figure 4 shows the *RsaI* digestions for the eight SCN inbred lines and shows that all SCN inbred lines that contain the *Hg-cm-1B* form in the DNA gel blot have the *RsaI* restriction digestion site in their cDNA. This data confirms that the SCN inbred lines express distinct *Hg-cm-1A* and *1B* cDNAs and that this expression also correlates with SCN virulence. The observation that not all the *Hg-cm-1B* cDNA digests with *RsaI* indicates that the nematode produces a mixture of *Hg-cm-1* cDNA; this correlates well with the observation that most SCN lines contain both *Hg-cm-1A* and B forms but differ in copy number (Fig. 3).

DISCUSSION

Hg-cm-1 is the second functional nematode chorismate mutase isolated from plant parasitic nematodes. In addition, the chorismate mutase isolated from *Globodera pallida* (Jones et al. 2002; Popeijus et al. 2000a) is closely related and probably will be shown to have CM enzymatic activity. Since *Hg-cm-1* has strong homology to bacterial CMs, it was important to confirm its nematode origin. The observation that *Hg-cm-1* cDNA specifically hybridizes to nematode genomic DNA on DNA gel blots, the fact that the genomic clone of *Hg-cm-1* contains three introns, and the observation that the cDNA contains a SL-1 spliced leader sequence supports a nematode origin for *Hg-cm-1*. Overall, the *Hg-CM-1* protein has three domains, an N-terminal domain, a CM domain, and a C-terminal domain. Only the central CM region has a known function, and its activity is not dependent on the N-terminal domain. In contrast, *Mj-CM-1* only has a CM domain and a C-terminal domain of unknown function (Fig. 1). The main difference between the nematode CM domains is at amino acid residues 11 and 84. At amino acid 11, the cyst nematode CMs have lysine residues while *Mj-CM-1* has a functionally equivalent arginine at residue 20 (Fig. 1). In *Mj-CM-1* R20 is required for high levels of CM activity (K. Lambert, *personal communication*), while in

E. coli CM arginine 11 is essential for CM function, since an arginine to lysine mutation significantly lowers its activity (Liu et al. 1996). However, in some CMs, the arginine 11 residue is not conserved, so it is not clear if the arginine 11 to lysine change would affect *Hg-CM-1* activity (Xia et al. 1993) (Fig. 1). Serine 84 is considered an important active site residue in *E. coli* (Hur and Bruice 2002), but the asparagine residues in the cyst nematode CM's could also form the required hydrogen bond with chorismate. Given that *Hg-CM-1* has two changes in active site residues, it could be possible that it has different enzyme kinetics from that of *Mj-CM-1*. The amino acid changes between *Hg-CM-1A* and *Hg-CM-1B* do not change any of the CM active site residues, and two of the five amino acid changes are in the C-terminal domain. Therefore, it is not possible to predict any dramatic changes in enzymatic activity between these two forms of the enzyme. All nematode CMs will have to be analyzed further to determine if there are any measurable differences in their enzymatic properties.

The observation that *Hg-cm-1* is localized to the esophageal glands of the nematode and that the predicted protein contains a putative signal peptide supports the idea that the *Hg-CM-1* protein is secreted from the nematode during the onset of nematode parasitism. It is not clear from the *in situ* hybridizations whether *Hg-cm-1* is expressed in two or three of the nematodes esophageal glands, but expression in both the dorsal and subventral glands is consistent with that of *Mj-cm-1*. In addition, the *Mj-CM-1* protein is secreted from the nematode during the initiation of parasitism and is expressed by the nematode at high levels only during the onset of parasitism (Doyle and Lambert 2003; Painter and Lambert 2002). The similarity of the gene expression pattern of *Hg-cm-1* to *Mj-cm-1* suggests that both are secreted and regulated in a similar manner.

Potential functions of *Hg-CM-1*.

One assumption we make when discussing the role of nematode chorismate mutases in plant parasitism is that the CM is injected into the cytoplasm of the plant cell. This assumption is well founded, because CM is an enzyme in the plant's shikimate pathway, which is localized in the plastid. Interestingly, a branch of the shikimate pathway starting at chorismate is also found in the plant cytoplasm (Eberhard et al. 1996; Sommer and Heide 1998; Weaver and Herrmann 1997). The observation

Fig. 3. Genomic DNA gel blot of *EcoRI*-digested DNA extracted from inbred lines of *Heterodera glycines* hybridized with *H. glycines* chorismate mutase (*Hg-cm-1*). *H. glycines* inbred lines OP50, TN20, TN19, and TN15 grow on *H. glycines*-resistant soybeans PI90763 and PI89772 and, predominantly, have band B (2.2 kb), while *H. glycines* inbred lines VL1, TN16, TN3, TN2, and TN1, which do not grow well on PI90763 and PI89772 resistant soybeans, predominantly have band A (2 kb). The markers are shown in kilobase pairs.

that chorismate is utilized in both subcellular compartments but is only synthesized in the plastid sets up a situation in the plant cell in which they compete for a common chorismate source. This biochemical competition suggests a potential model for how a nematode CM could manipulate plant metabolism. If an unregulated nematode CM is introduced into this highly regulated pathway, it could tip the balance in favor of the cytosolic branch of the pathway, outcompeting the plastid pathway for chorismate and suppressing the formation of plastid chorismate-derived compounds (Fig. 5). In addition to the aromatic amino acids, chorismate-derived compounds include the plant hormone indole-3-acetic acid (Bartel 1997), the plant defense signal compound salicylic acid (Metraux 2002; Wildermuth et al. 2001), plant defense compounds like phenolic phytoalexins (Hammerschmidt 1999; Maher et al. 1994), and cell wall components such as lignin (Whetten and Sederoff 1995) and many others. In support of this "chorismate competition model," it has been shown that the overexpression of *Mj-CM-1* in soybean hairy roots suppresses the formation of indole-3 acetic acid (Doyle and Lambert 2003). This observation suggests that suppression of plant defense compounds like salicylic acid and phenolic phytoalexins derived from chorismate could also occur, suppressing host plant resistance. Salicylic acid is known to be essential for the function of plant resistance genes and for systemic acquired resistance mechanisms (Ryals et al. 1996), while phenolic phytoalexins are known to be toxic to plant nematodes (Gonzalez and Estevez-Braun 1997; Kaplan et al. 1980). In this paper, we present correlative evidence for the involvement of *Hg-cm-1* in the suppression of plant resistance to SCN in soybean. The restriction fragment polymorphisms in the DNA gel blot hybridized with *Hg-cm-1* cDNA clearly show a correlation with the *Hg-cm-1* restriction pattern and the ability of the nematode to parasitize resistant soybean cultivars PI90763 and PI89772. The same polymorphism is seen in the *Hg-cm-1* cDNA when the SCN inbred lines are compared, indicating the SCN inbred lines do express different *Hg-cm-1* forms. The fact that many inbred nematode lines originate from different geographical regions of the United States suggests that the *Hg-cm-1* restriction fragment pattern is not simply due to relatedness of the inbred lines but may be due to a selection of the *Hg-cm-1B* form that provides the nematode an increased ability to parasitize some resistant soybean plants. While little is known about how soybean resistance to SCN can be overcome, it seems possible that *Hg-CM-1* could suppress defense compounds like salicylic acid, phenolic phytoalexins, or another essential chorismate-derived compound that would aid the nematode in breaking down host plant resistance. It seems reasonable that plant parasitic nematodes may produce different forms of CMs that vary in copy number, enzymatic activity, or level of expression. Such differences are reflected in the polymorphisms we see between in-

bred SCN and suggest one or more mechanisms for why some SCN have an increased ability to grow on some resistant soybean plants. Further characterization of the *Hg-cm* gene family will have to be conducted to measure the differences among *Hg-cm* family members and to correlate these differences with the ability of SCN to grow on resistant soybean cultivars. In addition, genetic crosses between SCN with *Hg-cm-1A* and *-1B* will have to be conducted to show segregation of *Hg-cm-1B* with the ability to grow on PI90763 and PI89772.

While the *Hg-cm-1* polymorphisms we see between SCN inbred lines correlate with the nematodes ability to break host plant resistance on PI90763 and PI89772, it does not explain SCN ability to parasitize all SCN resistant soybean varieties. This observation is consistent with genetic data that show that SCN contains multiple virulence genes; *ror-1*, *ror-2*, *kr3*, and *kr4* have been genetically characterized by Dong and Opperman (1997), and there are probably many more yet to be discovered. More SCN virulence genes will have to be cloned and analyzed to fully explain the molecular biology of SCN virulence.

The ability to predict SCN virulence using *Hg-cm*-type or other virulence genes could prove to be extremely valuable in controlling SCN in the field. The ability to predict SCN virulence or the virulence of any nematode using a DNA-based assay would be a boon for growers throughout the world. In the

Fig. 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) restriction fragment linked polymorphism analysis on *Hg-cm-1* cDNA. The ethidium bromide-stained agarose gel shows PCR-amplified *Hg-cm-1* cDNA from eight SCN lines digested with the restriction enzyme *RsaI*. *H. glycines* inbred lines VL1, TN16, TN2, and TN1, which do not grow well on PI90763 and PI89772 resistant soybeans, express *Hg-cm-1A* that does not digest with *RsaI*, while *H. glycines* inbred lines OP50, TN20, TN19, and TN15 grow well on *H. glycines*-resistant soybeans PI90763 and PI89772 and express *Hg-cm-1B* that cuts with *RsaI*. The markers are shown in kilobase pairs.

Table 2. Virulence pattern of *Heterodera glycines* inbred lines on soybean plant introductions (PI)^a

Inbred lines	Plant resistance to <i>Heterodera glycines</i>						
	PI548402	PI88788	PI90763	PI437654	PI209332	PI89772	PI548316
TN1	7	12	1	0	17	4	24
TN2	37	5	14	0	6	16	2
TN3	44	3	10	0	2	16	1
TN15	93	5	54	0	7	80	29
TN16	39	127	0	0	69	0	103
TN19	59	69	49	70	63	56	71
TN20	70	57	79	78	80	67	88
VL1	2	64	0	0	45	0	44
OP50	107	45	86	0	41	88	33

^a The numbers reported in the table are the female index, the percentage of growth on the *Heterodera glycines* resistant soybeans as compared with the susceptible control and indicate the extent that *H. glycines* grows on nematode-resistant soybean plants.

future, we hope to be able to predict some, but not all, SCN virulence using *Hg-cm* types. Further research on the *Hg-cm-1* gene family and that of other virulence genes may well lead us into a new era of SCN virulence testing and management.

MATERIALS AND METHODS

Inbred lines, culture, and characteristics.

A collection of SCN inbred lines selected by single-cyst descent or mass selection on soybean lines is maintained at 27°C in a greenhouse facility on appropriate host plants using plastic crocks as described by Niblack and associates (1993). All SCN lines were isolated in the United States: TN1, TN2, TN3, TN15, and TN16 are from Missouri; TN19 and TN20 are from Tennessee; OP50 is from North Carolina (Dong and Opperman 1997); and VL1 was selected from a mixed population collected throughout the entire United States (Luedders 1985).

Nematode culture and isolation of parasitic nematode stages.

SCN cDNA was amplified from seven nematodes that were extracted from the root four days after inoculation. The nematode SCN used for the library construction was a race 3 Illinois field population. SCN were grown and extracted as described by Niblack and associates (1993). Eggs were then hatched at 26°C and 50 ± 5 second-stage juveniles were inoculated into soybean seedlings growing in 50-ml plastic tubes (*Glycine max* (L.) Merr cv. Essex) filled with river sand and were incubated in a growth chamber with continuous light at 22 to 25°C. Parasitic nematode stages were retrieved from infected roots by tearing the root in half with forceps and dislodging the nematodes from the root tissue, using an electrically-etched tungsten knife (Cutter 1967). The SCN were transferred to a 0.2-ml PCR tube containing a proteinase K solution using the tungsten knife.

cDNA amplification and library construction.

RNA was extracted from nematode tissue in 10 µl of proteinase K solution (0.1 M Tris-HCl (pH 8.0), 12.5 mM EDTA, 0.15 M LiCl, 1% sodium dodecyl sulfate, 1 mM dithiothreitol, 800 ng 5S RNA, 2 mg ml⁻¹ proteinase K) for 1 h at 37°C. After

extraction, 15 µl of diethylpyrocarbonate-treated H₂O was added to the proteinase K digest, and the RNA was denatured at 100°C for 2 min. The cDNA was synthesized and amplified, as described by Lambert and associates (1999).

For the cDNA polymorphism experiments, RNA was extracted as described above but with the inclusion of an overnight RNA precipitation in 2 M LiCl at 4°C. The RNA was dissolved in nuclease-free water and then precipitated with ethanol overnight at -20°C. Precipitated RNA was dissolved in nuclease-free water, and trace amounts of genomic DNA were removed using the DNA-free kit (Ambion, Austin, TX, U.S.A.). The cDNA was synthesized from 2 µg of total RNA using oligo dT and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, U.S.A.). The *Hg-cm-1* cDNA was PCR-amplified from the SCN cDNA using primers SCNCM-5F and SCNCM-5R at 94°C (30 s), (94°C [30 s], 51°C [30 s], 68°C [1 min], 30 cycles), and 68°C (30 min). The PCR products were digested with the restriction endonuclease RsaI, and the digestion products were fractionated on a 2% agarose gel, were stained with ethidium bromide, and were photographed using the Kodak Electrophoresis Documentation System (Kodak, Rochester, NY, U.S.A.).

Oligonucleotides.

Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.):

CM-deg: 5'-TTTRYTAGCRTYYATTTGGTCTTG-3'

SL: 5'-CTCGCTCGCCCAGGTTTAATTACCCAAGTTTAAG-3'

HGCM-3: 5'-AAGCAAACCGATGGGCCAATGCAAT-3'

LMOD: 5'-CTGGTTCGGCCACATTGACGTCGACTATCCA-3'

HGCM5F: 5'-GCCATGGGACATGCGAGAAACATTGCAC-3'

HGCM6F: 5'-GCCATGGGAATGAAATGGCCAAACG-3'

HGCM5R: 5'-GGCCAACAATTTCTTTGC-3'.

Cloning of the *Hg-cm-1* cDNA.

Using degenerate primer CM-deg in combination with the SL primer and the cDNA produced from four-day-old parasitic juvenile SCN, we PCR-amplified the 5' end of *Hg-cm-1*. PCR was performed under the following conditions: 94°C (30 s), (94°C [30 s], 60°C [30 s], 68°C [1 min], 30 cycles), and 68°C for 10 min, using KlenTaq polymerase and supplied buffer (Ab Peptides, St. Louis). The PCR product of the *Hg-cm-1* cDNA was cloned into the pCRII-TA TOPO vector (Invitrogen) and was sequenced. To obtain the 3' end of the *Hg-cm-1* cDNA clone, specific primer SCN-CM-3 derived from the initial sequence was used in combination with the polyA tail linked LMOD primer. PCR was run in the same conditions as above, except for 65°C annealing temperature. The amplified PCR product was also cloned in pCRII-TA TOPO vector and was sequenced. The *Hg-cm-1* genomic clone was PCR-amplified from the SCN genomic DNA isolated from SCN line TN19, using primers SCNCM-5F and SCNCM-5R at 94°C (30 s), (94°C [30 s], 51°C [30 s], 68°C [1 min], 30 cycles), and 68°C (30 min). The PCR product was cloned into the pCRII-TA TOPO vector and was sequenced.

DNA sequence analysis.

All cDNA clones were sequenced at the University of Illinois Biotechnology Center. BLASTP2 was used for sequence similarity searches (Altschul et al. 1997). Chorismate mutase protein sequences were aligned using CLUSTAL W, version 1.6 (Thompson et al. 1994). Secondary structure prediction was calculated for the aligned sequences by the Predict Protein package (Rost and Sanders 1995).

DNA gel blotting.

Eggs and preparasitic second-stage juvenile nematodes were collected from greenhouse nematode cultures. Packed

Fig. 5. Chorismate competition model for chorismate partitioning. Diagram of a plant cell depicting the subcellular location of the shikimate pathway and phenylalanine, tyrosine, and tryptophan biosynthesis. E4P = erythrose 4-phosphate, PEP = phosphoenolpyruvate, CM = chorismate mutase, IAA = indole-3-acetic acid, and SA = salicylic acid. The dark arrows indicate the flow of metabolites through the shikimate pathway when *Heterodera glycines* chorismate mutase (*Hg-CM-1*) is introduced into the plant cell cytoplasm.

nematodes (100 µl) were frozen in liquid nitrogen and then broken to small pieces in a tissue pulverizer (Fisher Scientific, Pittsburgh, PA, U.S.A.). The frozen nematode fragments were mixed with 1 ml of a proteinase K solution and were incubated at 37°C for 1 h, as described above. The DNA was phenol/chloroform-extracted and then precipitated with ethanol (Sambrook et al. 1989). The resuspended genomic DNA was treated with RNase (10 µg ml⁻¹) at 37°C. Soybean DNA was extracted from leaves of Essex soybean. Soybean DNA (20 µg) and nematode genomic DNA (10 µg) was used for each lane of DNA gel blot. Nematode DNA of SCN inbred lines and soybean was digested with *EcoRI* and was transferred to a nitrocellulose membrane. *Hg-cm-1* cDNA was purified with a Chromaspin 100 column (Clontech, Palo Alto, CA, U.S.A.) and then random prime labeled with P³²dCTP with the Prime-it II kit (Stratagene, La Jolla, CA, U.S.A.). The probe was hybridized to the membrane and was detected by standard protocols (Sambrook et al. 1989). Blots were typically exposed to X-ray film for 3 days at -80°C with one intensifying screen.

In situ hybridization.

A small cDNA PCR product of 130 bp was amplified as above with specific *Hg-cm-1* primers (CM-2F and CM-2R) and was cloned in pCRII TOPO vector and used for in situ hybridization probe template. The cDNA insert was purified with a Chromaspin-200 column. Digoxigenin-labeled RNA probes were synthesized from 300 ng of purified template with T7 and T3 AmpliScribe High Yield Transcription Kits (Epicentre Technologies, Madison, WI, U.S.A.) with the following nucleotide concentrations: 7.5 mM GTP, 7.5 mM ATP, 7.5 mM CTP, 5 mM UTP, 3 mM dig-11-UTP (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Labeled RNA was purified with a Chromaspin-200 column. The probe was used at a concentration of 1 ng µl⁻¹. Excised nematode-infected root tips were fixed in FAA (3.7% formaldehyde, 50% ethanol, 5% glacial acetic acid) and were paraffin embedded (Schichnes et al. 1999), and 10-µm sections were cut on a rotary microtome. The paraffin sections were processed and hybridized to the *Hg-cm-1* riboprobes, according to published protocols (Jackson 1992). The hybridized probe was detected following the protocol of Coen and associates (1990) with slight modifications (Ruzin 1999). Labeled cells were examined using a Zeiss Axioscope 2 microscope, and images were captured using a Zeiss AxioCam cooled color CCD camera.

Bacterial expression of *Hg-cm-1*.

The *Hg-cm-1* coding region minus the putative signal sequence was amplified by PCR, using primers SCNCM-5F and SCNCM-5R at 94°C (30 s), (94°C [30 s], 51°C [30 s], 68°C [1 min], 30 cycles), and 68°C (30 min). The *Hg-cm-1* coding region minus the N-terminal domain was amplified using primers SCNCM-6F and SCNCM-5R, using the PCR conditions described above. The resulting PCR products were inserted into pBAD/Thio-TOPO plasmid expression vector and then transformed into *E. coli* JP2261 (*aroF363*, *pheO352*, *pheA365*, *tyrA382*, *thi-1*, *lac*, *tsx-358*, *rpsL712*, and *xyl*) (Baldwin and Davidson 1981). Transformed *E. coli* cells were plated on Luria-Bertani (LB) agar media containing 100 µg of ampicillin per ml. Colonies containing the *Hg-cm-1* in the forward direction were grown overnight in LB media supplemented with 100 µg of ampicillin per ml. The overnight cultures of *E. coli* harboring *Hg-cm-1* were diluted in LB media plus 100 µg of ampicillin per ml and were grown to an optical density at 600 nm of 0.5 to 0.6, and then, arabinose was added to a final concentration of 0.02%, and the culture was allowed to grow 2 h more at 25°C.

Chorismate mutase enzyme assay.

E. coli cells expressing *Hg-CM-1* were concentrated by centrifugation at 4°C and were lysed by the freeze-thaw method. Six histidine-tagged proteins were purified with talon metal affinity resin (Clontech). The *E. coli* cells were lysed, and the proteins were purified on the talon resin, following manufacturer's protocols. All protein extracts were assayed for CM activity with the enol-borate complex method (Gilchrist and Connelly 1987).

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