Selection of *Heterodera glycines* Chorismate Mutase-1 Alleles on Nematode-Resistant Soybean

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The soybean cyst nematode *Heterodera glycines* is the most destructive pathogen of soybean in the United States. Diversity in the parasitic ability of the nematode allows it to reproduce on nematode-resistant soybean. *H. glycines* chorismate mutase-1 (*Hg-CM-1*) is a nematode enzyme with the potential to suppress host plant defense compounds; therefore, it has the potential to enhance the parasitic ability of nematodes expressing the gene. *Hg-cm-1* is a member of a gene family where two alleles, *Hg-cm-1A* and *Hg-cm-1B*, have been identified. Analysis of the *Hg-cm-1* gene copy number revealed that there are multiple copies of *Hg-cm-1* alleles in the *H. glycines* genome. *H. glycines* inbred lines were crossed to ultimately generate three F₂ populations of second-stage juveniles (J2s) segregating for *Hg-cm-1A* and *Hg-cm-1B*. Segregation of *Hg-cm-1A* and *1B* approximated a 1:2:1 ratio, which suggested that *Hg-cm-1* is organized in a cluster of genes that segregate roughly as a single locus. The F₂ *H. glycines* J2 populations were used to infect nematode-resistant (Hartwig, PI88788, and PI90763) and susceptible (Lee 74) soybean plants. *H. glycines* grown on Hartwig, Lee 74, and PI90763 showed allelic frequencies similar to *Hg-cm-1A/B*, but nematodes grown on PI88788 contained predominately *Hg-cm-1A* allele as a result of a statistically significant drop of *Hg-cm-1B* in the population. This result suggests that specific *Hg-cm-1* alleles, or a closely linked gene, may aid *H. glycines* in adapting to particular soybean hosts.

Additional keywords: parasitism gene, resistance, shikimate pathway.

The soybean cyst nematode *Heterodera glycines* is the most destructive plant pathogen of soybean in the United States, causing billions of dollars in losses annually (Niblack 2004; Wrather et al. 2001). Diversity in the parasitic ability of *H. glycines* on resistant soybean was recognized soon after the nematode's discovery in North Carolina, when two Japanese *H. glycines*-resistant soybean cultivars were deemed susceptible to local *H. glycines* populations during field evaluations of resistance (Ross and Brim 1957). Subsequently, populations from Tennessee and North Carolina that differed in parasitism of resistant soybean plant introduction (PI) 88788 were named physiological strains (Ross 1962) and, as other populations were identified, the term "race" was adopted to categorize differences in parasitism among populations of *H. glycines*

(Golden et al. 1970; Ross 1962). Parasitism was assessed as a phenotypic response of numbers of females that developed on a set of differential *H. glycines*-resistant soybean lines (Riggs and Schmitt 1988). At present, *H. glycines* parasitism is designated by HgType, in which parasitism is assessed on a set of seven *H. glycines*-resistant soybean PIs used to develop cultivars or germ plasm lines resistant to *H. glycines* (Niblack et al. 2002). Although many *H. glycines*-resistant cultivars are available (Concibido et al. 2004), the resistance often is not durable and *H. glycines* adapts to and grows on the resistant plants, making plants or cultivars with resistance from those sources vulnerable (Colgrove et al. 2002; Koenning and Barker 1998; Noel and Edwards 1996; Schmitt and Barker 1987). HgType bioassays are useful for making cultivar recommendations to growers for specific *H. glycines*-infested fields, but they are time consuming to conduct. Knowledge of genes that govern *H. glycines* parasitism of resistant soybean, which also is referred to as virulence, will allow nematode populations to be quickly assessed for parasitic ability, aid breeders in developing new resistant soybean cultivars to control *H. glycines* in the field, and lead to an understanding of the underlying molecular basis for pathogenic diversity in *H. glycines*.

Genetic analysis has demonstrated that parasitism in cyst nematodes may be regulated by one or more genes that control the ability of *H. glycines* to overcome host-plant resistance, but these genes have not yet been cloned (Dong and Opperman 1997; Janssen et al. 1991; Triantaphyllou 1975). Root-knot and cyst nematodes express a variety of putative parasitism genes in their esophageal glands (Davis et al. 2000; Gao et al. 2002, 2003; Huang et al. 2003; Qin et al. 2000; Scholl et al. 2003; Wang et al. 2001), although it is not known if they enhance parasitic ability. Recently, putative avirulence genes expressed in the amphids and esophageal glands of the root-knot nematode were isolated, but their biochemical mode of action remains to be determined (Neveu et al. 2003; Semblat et al. 2001).

In addition to parasitism genes, plant nematodes also may produce small metabolites, such as Nod factors, that also could influence nematode parasitism (Scholl et al. 2003). Although nematode cellulases, pectate lyases, expansins, polygalacturonase, and chorismate mutases are the only putative parasitism genes that encode proteins with known activities (Doyle and Lambert 2002; Jaubert et al. 2002; Lambert et al. 1999; Popeijus et al. 2000; Qin et al. 2004; Smant et al. 1998), nematode chorismate mutase (NCM) is intriguing because it has a function that suggests it could play a role in directly altering host metabolism and plant defense mechanisms.

Chorismate mutase is an enzyme in the shikimate pathway, a primary metabolic pathway found in plants, bacteria, fungi,

and some protozoans (Roberts et al. 1998; Schmid and Amrhein 1995). The shikimate pathway does not exist in animals, which makes the expression of a secreted form of NCM by the root-knot (Doyle and Lambert 2003; Lambert et al. 1999) and potentially secreted forms by the soybean cyst (Bekal et al. 2003; Gao et al. 2003) and potato cyst (Jones et al. 2002; Popeijus et al. 2000) nematodes especially interesting. The shikimate pathway is vital in plants because its products include the aromatic amino acids (phenylalanine, tyrosine, and tryptophan), plant hormones (indole-3 acetic acid), cell wall

components (lignin), and numerous secondary metabolites that have plant defense activities (e.g., salicylic acid and phytoalexins) (Dewick 1998). It has been proposed that NCM is injected into the plant cell's cytoplasm where it alters the plant's shikimate pathway (Bekal et al. 2003; Doyle et al. 2003; Lambert et al. 1999). The observation that NCM expression in soybean hairy roots suppresses the formation of plant vascular tissue, presumably by suppressing auxin biosynthesis, supports this hypothesis (Doyle and Lambert 2003). The *H. glycines* chorismate mutase-1 (*Hg-cm-1*) is a member of a polymorphic gene family, of which two different forms have been cloned (*Hg-cm-1A* and *Hg-cm-1B*). *Hg-cm-1* is polymorphic in DNA gel blots between inbred *H. glycines* lines that differ in parasitism on HgType indicator lines (Bekal et al. 2003). Upon inbreeding of *H. glycines* on different resistant soybean lines, it was noticed that either *Hg-cm-1A* or *Hg-cm-1B* was selected (Bekal et al. 2003). Taken together, these observations have led to the hypothesis that *Hg-cm-1* may play a role in altering host defense responses elicited by nematode infection.

This article describes our continuing work to determine whether *Hg-cm-1A* or *Hg-cm-1B* is associated with the ability of specific *H. glycines* populations to reproduce on nematode-resistant soybean lines. To accomplish this objective, we conducted controlled crosses of inbred *H. glycines* and analyzed *Hg-cm-1* genotypes in the resulting F₂ *H. glycines* populations grown on resistant and susceptible soybean. To aid in the explanation of our cross data, we also analyzed the structure of the *Hg-cm-1* gene family.

RESULTS

Development of an *Hg-cm-1* genotype assay.

Because we were interested in conducting controlled crosses of inbred *H. glycines*, we needed to develop a reliable method to determine the *Hg-cm-1* genotype of individual nematodes throughout the crossing process. To conduct our genotyping, a real-time quantitative polymerase chain reaction (QPCR) single-feature polymorphism (SFP) assay was developed to simultaneously detect and quantify both *Hg-cm-1A* and *1B*. We refer to this assay as an SFM assay and not a single-nucleotide polymorphism assay because there are two nucleotide differences between *Hg-cm-1A* and *1B* that are used to discriminate between the two alleles during QPCR amplification.

Genomic DNA isolated from bulk *H. glycines* eggs of inbred lines TN16, VL1, TN19, and TN20 was tested using the *Hg-*

Fig. 1. Real-time polymerase chain reaction (PCR) amplification logarithmic plots for the *Heterodera glycines* chorismate mutase 1 single-feature polymorphism assay (*Hg-cm* SFP). In all plots, the X axis shows the number of PCR cycles and the Y axis shows the normalized fluorescence of the reporter dye (delta RN). *Hg-cm-1A* amplification curves are labeled "A" and *Hg-cm-1B* curves are labeled "B". The threshold cycle (C_T) is the cycle number where the amplification signal crosses a threshold line (T) and the ΔC_T is the difference in C_T between *Hg-cm-1* alleles. **A** through **D**, Amplification curves for the *Hg-cm* SFP assay conducted on genomic DNA purified from eggs of inbred *H. glycines*. Amplification curves for **A**, TN19 DNA, **B**, TN16 DNA, **C**, TN20 DNA, and **D**, VL1 DNA. The ΔC_T for TN19 and TN20 is 2.5, which is a 5.9-fold difference in *Hg-cm-1B* to *1A* based on the equation fold difference = $2^{-\Delta C_T}$ (Livak and Schmittgen 2001). **E**, Combined assay results for 12 individual second-stage juveniles (J2s) assayed from TN19 and 12 from TN20. **F**, Results of 12 individual J2s assayed from TN16 and 12 from VL1. **G**, Assay results of 24 F₁ J2s from the cross TN20 $\times$ TN16. **H**, Results of assaying 24 F₁ individual J2s from the cross TN19 $\times$ VL1. Because all *H. glycines* F₁ individuals had both *Hg-cm-1A* and *1B* signals, and these signals could not be individually labeled, they were not marked on the amplification plot.

cm SFP assay. Only *Hg-cm-1A* allele was detected in *H. glycines* TN16 and VL1 (Fig. 1). The signal from the *Hg-cm-1B* allele never exceeded the threshold line; therefore, no significant cross-amplification of *Hg-cm-1A* and *1B* occurred (Fig. 1). When *H. glycines* TN19 and TN20 were tested with the *Hg-cm* SFP assay, *Hg-cm-1B* was the predominant allele detected; however, *Hg-cm-1A* also was detected in TN19 and TN20 at a sixfold lower abundance than *Hg-cm-1B* allele (Fig. 1).

To be useful, the *Hg-cm-1* SFP assay had to be able to genotype *H. glycines* individuals; therefore, we assayed individual second-stage juveniles (J2s) of inbred lines TN16, VL1, TN19, and TN20. The assay detected only *Hg-cm-1A* in inbred line TN16 and VL1 individuals, but detected mainly *Hg-cm-1B* and a lesser amount of *Hg-cm-1A* in TN19 and TN20 individuals (Fig. 1). In F₁ individuals from crosses between *Hg-cm-1A* and *1B* type parents, both alleles showed robust signals (Fig. 1). Collectively, this data indicates that our *Hg-cm* SFP assay could be used to genotype individual nematodes and is reliable and accurate.

Controlled crosses of inbred *H. glycines*.

Three crosses between *H. glycines* inbred lines were conducted: TN19 (female) × VL1 (male), TN20 (female) × TN16 (male), and TN16 (female) × TN20 (male), using a modification of the *H. glycines* crossing method described by Dong and Opperman (1998). A subset of the F₁ J2 individuals were genotyped using the *Hg-cm* SFP assay to be sure that they were heterozygous. Plots of the *Hg-cm* SFP assay data for two hybrids, TN20/TN16 and TN19/VL1, showed that both *Hg-cm-1A* and *1B* signals were strong in all F₁ *H. glycines* (Fig. 1). Genotyping indicated that the crossing method was efficient in producing viable F₁ progenies and showed very limited sibling mating (below 5%) (Table 1). The F₁ *H. glycines* J2 individuals were allowed to infect susceptible soybean plants (cv. Lee) where they randomly mated for one generation. A subset of the resulting F₂ J2 individuals were genotyped. The results indicate *Hg-cm-1A* and *1B* segregated in a 1:2:1 ratio for two of the three crosses (Table 2).

The F₂ *H. glycines* J2 population was split into four equal groups and grown on susceptible soybean cv. Lee and three resistant soybean plants, PI 88788, PI 90763, and cv. Hartwig. The resulting virgin females were harvested from each plant and individually genotyped for *Hg-cm-1A* and *1B*. When the frequency of each genotype in an *H. glycines* population was compared between the nematodes grown on resistant and susceptible plants, a consistent statistically significant increase in *Hg-cm-1A* genotype was seen in nematode populations grown on resistant soybean PI88788 compared with susceptible Lee (Table 3). None of the other selection experiments showed a consistent preference for *Hg-cm-1A* or *1B* among all three crosses (data not shown). However, when the copy number of *Hg-cm-1A* and *1B* for each selected population of *H. glycines* was compared, a consistent decrease in *Hg-cm-1B* was observed in all three crosses, as noted by an increase in *Hg-cm-1A/1B* ratio for *H. glycines* grown on PI88788 (Table 4). These data showed that a negative selection of a locus linked to or *Hg-cm-1B* itself was responsible for the predominance of *Hg-cm-1A* genotype. This trend in the decrease of *Hg-cm-1B* was statistically significant and observed in all crosses (Table 4). This analysis allows for the evaluation of the association between resistance and *Hg-cm* type while controlling for each experimental run or cross in a manner similar to blocking in a two-way analysis of variance. The Mantel-Haenszel statistic indicates that there was a general trend toward decreased presence of the *Hg-cm-1B* allele under PI88788 resistance, more so than would be expected by chance alone. This trend was reflected in each individual cell in the table as well.

Gene structure of the *Hg-cm-1* gene family.

The observation that individual J2s had both *Hg-cm-1A* and *1B* in a ratio other than 1:1 suggests that *Hg-cm-1* is repeated in the *H. glycines* genome. To get a better idea of how many *Hg-cm* genes are in the *H. glycines* genome, DNA gel blots were conducted on *Eco*R1-digested *H. glycines* genomic DNA from the four parental inbred lines, TN16, VL1, TN20, and TN19. The blots were hybridized with an *Hg-cm-1B* cDNA probe (Fig. 2). When genomic DNA from TN16 and VL1 were digested completely, two main hybridizing bands (1.8- and 3.25-kb *Hg-cm-1A* alleles) indicated the presence of two *Hg-cm-1*-like genes in TN16 and VL1 (Fig. 2, lane 1). The *Hg-cm-1A* genomic clone and cDNA did not contain *Eco*R1 restriction sites in the region of the probe; therefore, the 3.25-kb band must represent another *Hg-cm-1* gene related to *Hg-cm-1A*. In TN16, we consistently observed a faint 5.2-kb band that may represent an undigested repeat because it is equal in size to one of the partially digested fragments. When genomic DNA from TN16 and VL1 was digested partially, at least six doublets of restriction fragments increasing in a consistent size from 3.25 kb to more than 10 kb were observed, suggesting that these genes are organized as tandem repeats.

When genomic DNA of TN19 and TN20 were digested completely, a major 1.9-kb band corresponding to *Hg-cm-1B* and three weaker bands corresponding to *Hg-cm-1A* were observed on autoradiographs (Fig. 2, lane 1). When genomic DNA of TN19 and TN20 were partially digested, we again saw a repeated pattern of restriction fragments increasing in size, but this time the doublet of bands was spaced farther apart than with TN16 and VL1 (Fig. 2). TN19 and TN20 showed at least six bands in the partially digested lanes, also suggesting that these genes are repeated in the *H. glycines* genome.

To gain further evidence that *Hg-cm-1* is repeated in the *H. glycines* genome, we devised a PCR-based approach. We reasoned that, if *Hg-cm-1* was repeated in the genome, the repeats were close together, and in the same orientation; therefore, PCR primers designed to amplify across the span of the repeated genes would be able to amplify the 5' end of one repeat, the intervening sequence, and the 3' end of an adjacent repeat (Fig. 2B). Genomic DNA from TN19, TN20, VL1, and TN16 was PCR amplified using *Hg-cm* repeat spanning primers and the PCR amplification products were run on an agarose gel,

Table 1. *Hg-cm-1* genotype of F₁ soybean cyst nematode (SCN) second-stage juveniles (J2s)^a

SCN cross	<i>Hg-cm-1A</i>	<i>Hg-cm-1B</i>	<i>Hg-cm-1A/1B</i>
TN19 (female) × VL1 (male)	4/83	1/83	78/83
TN20 (female) × TN16 (male)	3/84	3/84	78/84
TN16 (female) × TN20 (male)	0/84	1/84	83/84

^a Individual *Heterodera glycines* J2s were genotyped for *Hg-cm-1A* and *Hg-cm-1B* using the *Hg-cm* single-feature polymorphism assay. The number of *H. glycines* J2s that scored positive for *Hg-cm-1A* or *Hg-cm-1B* is listed first, followed by the total number of individuals genotyped.

Table 2. *Hg-cm-1* genotype of F₁ soybean cyst nematode (SCN) second-stage juveniles (J2s)^a

SCN cross	<i>Hg-cm-1A</i>	<i>Hg-cm-1B</i>	<i>Hg-cm-1A/1B</i>
TN19 (female) × VL1 (male)	18/82	18/82	46/82 ^b
TN20 (female) × TN16 (male)	12/81	13/81	56/81
TN16 (female) × TN20 (male)	17/75	11/75	47/75 ^b

^a Individual *Heterodera glycines* J2s were genotyped for *Hg-cm-1A* or *Hg-cm-1B* using our *Hg-cm* single-feature polymorphism assay. The data is reported as number of *H. glycines* J2s containing *Hg-cm-1A* or *Hg-cm-1B* per total number of individuals assayed.

^b The χ^2 distribution fits a 1:2:1 ratio with $P < 0.05$.

blotted to nitrocellulose, then hybridized with an *Hg-cm-1* cDNA probe (Fig. 2C). The probe hybridized to a 3.2-kb PCR product in all reactions, but with TN16 and VL1 having a stronger hybridization signal. The 3.2-kb amplification product from TN16 was cloned and sequenced, revealing 209 bp of the 3' end of *Hg-cm-1A*, a 1.5-kb intervening sequence between the two genes, and a 455-bp portion of the 5' end of a gene with strong similarity to *Hg-cm-1* (accession number AY879077), which we will refer to as *Hg-cm-2*. *Hg-cm-1* and *Hg-cm-2* are identical in the chorismate mutase domain, but are only 41% identical at a nucleotide level in the region of the gene that encodes the N-terminal domain of *Hg-cm-1* (Bekal et al. 2003). Because the PCR product had the structure predicted in Figure 2B, this experiment provides further evidence of the repeated nature of the *Hg-cm* gene family, but reveals sequence divergence between the genes.

DISCUSSION

Previously, we suggested that *Hg-cm-1A/1B* might aid *H. glycines* in parasitizing nematode-resistant soybean. We formed this hypothesis because restriction fragment length polymorphisms were observed between *H. glycines* nematodes that differed in their pattern of parasitism on resistant soybean plants and also because the chorismate mutase activity of *Hg-cm-1* might alter the plant's shikimate pathway to suppress chorismate-derived compounds that are defense related (Bekal et al. 2003). To follow up on this observation, we used a population genetic approach to investigate the association of *Hg-cm-1* with the parasitic ability of *H. glycines* on nematode-resistant soybean.

Hg-cm SFP assay validation.

The first step in our genetic analysis was to develop an *Hg-cm* SFP assay to track *Hg-cm-1A* and *1B* alleles during the crosses. This assay was critical for our experiments; therefore, care was taken to validate its effectiveness. Genotyping individual plant-parasitic nematodes is a technical challenge due to their small size, but PCR-based genotyping has been used successfully for plant-parasitic nematode diagnostics and phylogenetic studies targeting mitochondrial and ribosomal DNA

(Harris et al. 1990; Thomas et al. 1997). In our *Hg-cm* SFP assay, we employed real-time PCR because it is quantitative, very sensitive, and can be used for high-throughput genotyping. In developing the *Hg-cm* SFP assay, it needed to be able to specifically detect both *Hg-cm-1A* and *1B* without significant cross-reaction between the genes, and accurately and reproducibly genotype *Hg-cm-1* in single nematodes. Initial tests with *H. glycines* genomic DNA extracted from bulk preparations of eggs indicated that our *Hg-cm* SFP assay could detect and quantify each *Hg-cm* allele in a complex mixture. Individual parental *H. glycines* nematodes proved amenable to the *Hg-cm* SFP assay and showed similar amounts of *Hg-cm-1A* and *1B* compared with the purified pooled genomic DNA (Fig. 1). Detection of *Hg-cm-1* alleles in individual nematodes differed from bulked DNA samples in two respects: i) there was more sample-to-sample variation in individuals than with bulked genomic DNA and ii) in individuals from parental lines TN19 and TN20, where both *Hg-cm-1* alleles were in the nematode, the less-abundant allele often appeared lower in abundance than in the genomic DNA purified from nematode eggs. The variation in *Hg-cm-1A* and *1B* signal probably was due to differing amounts of nematode lysate, or released DNA, being added to the well because the nematodes were simply squashed, then added to the PCR tubes. The under-amplification of the less-abundant allele almost certainly was due to intrareaction competition for PCR resources (primers, nucleotides, and enzyme) between the more- and less-abundant alleles, a common phenomena in multiplex PCR (Freeman et al. 1998). However, because we saw this intrareaction competition phenomena only in nematodes with *Hg-cm-1A* or *1B* abundances similar to those of parental *H. glycines* TN19 and TN20 and not in the F₁ heterozygous nematodes, this effect did not significantly impact our data, because it just slightly underestimated the copy number for the lower-copy number alleles in some nematodes in the population, but did so consistently in all samples. Therefore, we conclude that the *Hg-cm* SFP assay accurately genotypes individual nematodes and provides semi-quantitative data on the relative abundance of the two *Hg-cm-1* alleles in *H. glycines* populations. However, the *Hg-cm-1A* or *1B* copy number estimates for individual nematodes likely do not represent the actual number of copies per genome because

Table 3. *Hg-cm-1* genotypes of F₂ *Heterodera glycines* grown on selective soybeans^a

SCN Cross	Hg-cm-1A ^b	Hg-cm-1B	Hg-cm-1A/1B
TN19 (female) × VL1 (male) S	29/83	12/83	42/83
TN19 (female) × VL1 (male) R	49/82	12/82	21/82
TN20 (female) × TN16 (male) S	12/84	18/84	53/84
TN20 (female) × TN16 (male) R	21/84	8/84	55/84
TN16 (female) × TN20 (male) S	17/84	18/84	49/84
TN16 (female) × TN20 (male) R	33/84	7/84	44/84

^a Individual *H. glycines* virgin females were genotyped for *Hg-cm-1A* or *Hg-cm-1B* using our *Hg-cm* SFP assay. The data is reported as number of *H. glycines* containing *Hg-cm-1A* or *Hg-cm-1B* per total number of individuals; S and R indicate *H. glycines* grown on susceptible soybean (Lee) and resistant soybean PI88788, respectively.

^b A student *t* test indicates the increase in number of *H. glycines* females with a *Hg-cm-1A* genotype when they are grown on PI88788 is statistically significant (*P* < 0.05).

Table 4. Cochran-Mantel-Haenszel (CMH) statistic of *Hg-cm-1* selection^a

Cross ^b	S alleles			R alleles			χ^2	DF	Probability
	A	B	A/B	A	B	A/B			
1	207,902	19,069	10.9	155,835	8,042	19.3	1,799.9	1	<0.0001
2	207,464	36,558	5.5	204,240	21,415	9.5	3,267.5	1	<0.0001
2R	91,707	15,194	6.0	69,108	2,796	24.7	5,064.1	1	<0.0001
CMH	...	...	...	...	...	...	8,877.8	...	<0.0001

^a *H. glycines* nematodes grown on susceptible soybean Lee (S) and resistant soybean PI88788 (R) are listed. The total number of copies of *Hg-cm-1A* (A) and *Hg-cm-1B* (B) alleles and the ratio of *Hg-cm-1A* to *1B* alleles (A/B) from 82 individual virgin *H. glycines* is shown; DF = degrees of freedom.

^b Cross 1 is TN19 (female) × VL1 (male), cross 2 is TN20 (female) × TN16 (male), and cross 2R is TN16 (female) × TN20 (male).

of variation in the efficiency of DNA extraction and intrareaction competition; therefore, this data is useful only for comparisons between populations of nematodes.

Gene structure of the *Hg-cm-1* gene family.

Previously, we noticed in DNA gel blots that the hybridization intensity of *Hg-cm-1A* and *1B* differed within bulk preparations of nematode DNA (Bekal et al. 2003). The inbred nematodes should have been nearly homozygous at all loci;

therefore, we suspected that *Hg-cm-1* was repeated in the nematode genome. When we performed *Hg-cm* SFP analysis on individual TN19 or TN20 J2 nematodes, a similar difference in copy number of *Hg-cm-1A* and *1B* seen in the DNA gel blots was observed (Fig. 1). These experiments also suggested that *Hg-cm-1* was repeated in the *H. glycines* genome. The PCR experiments that amplified adjacent genes showed that at least two *Hg-cm* genes are repeated tandemly; similarly, the DNA-gel blot experiments with partially digested *H. glycines*

Fig. 2. A, DNA-gel blots of *Heterodera glycines* genomic DNA digested for differing amounts of time and hybridized with a ^{32}P *Hg-cm-1B* cDNA probe. DNA in lanes 1 through 4 was digested for 6 h and 45, 20, and 10 min, respectively, on each blot. Sizes of selected bands are shown in kilobases next to the autoradiographs. The two sets of three vertical lines between the blots mark the repeated doublet band pattern in lanes 2 through 4. **B,** Representation of the gene structure of the *Hg-cm* gene family and where *Hg-cm-1* repeat-spanning polymerase chain reaction (PCR) primers (represented by arrows) hybridize to the genes. The distance between the genes, in kilobases, is shown under a bracket. **C,** DNA gel blot autoradiogram of the PCR amplification products using the primers depicted in panel B. The blot was hybridized with a ^{32}P -labeled *Hg-cm-1B* cDNA probe. The size of the hybridizing band is shown in kilobases next to the blot.

genomic DNA also suggest that the *Hg-cm-1* genes are repeated tandemly in the *H. glycines* genome, but not in a simple repeat pattern. Based on the DNA gel blot data, we estimate that there are at least 12 copies of *Hg-cm-1* in each *H. glycines* genome. The exact number of repeats was difficult to determine because conventional agarose gel electrophoresis can resolve fragments only of less than 20 kb. The observation that *Hg-cm-1A* and *1B* segregate roughly in a 1:2:1 ratio also supports the idea that *Hg-cm-1* is organized as a tandemly arrayed block of genes that segregate as a single locus. The repeated gene organization of *Hg-cm-1* may be a method *H. glycines* uses to generate extra genetic diversity in their populations, thus maximizing their adaptability. In *Caenorhabditis elegans*, repeated genes typically generated sequence variation by incomplete crossover or gene conversion (Semple and Wolfe 1999) and this also may be happening in *H. glycines*.

Selection of *Hg-cm-1* alleles

on *H. glycines*-resistant and -susceptible soybean.

To determine whether *Hg-cm-1A* or *1B* was associated with the ability of *H. glycines* to parasitize resistant soybean, F_2 populations, segregating for *Hg-cm-1A* and *1B*, were generated and grown on resistant and susceptible soybean. The rationale for this experiment was that if *Hg-cm-1A*, *1B*, or a gene linked to *Hg-cm-1* assists the nematode in parasitizing a resistant plant, then one or the other *Hg-cm-1* allele should increase in frequency in the population. However, if neither *Hg-cm-1* helped the nematode parasitize the plant, then no change in the *Hg-cm-1A* or *1B* frequency should be observed between the selected populations. The observation that F_2 *H. glycines* J2s grown on a resistant soybean PI88788 were predominantly *Hg-cm-1A* type, which was due to a statistically significant drop in the abundance of *Hg-cm-1B* alleles in the population, suggests the *Hg-cm-1A* or a closely linked gene aids the nematode in parasitizing this type of soybean.

This data lends itself to several interpretations. One might be that *Hg-cm-1A* helps the nematode suppress host plant resistance of PI88788. Another explanation might be that *Hg-cm-1A* somehow aids the nematode in overcoming some other biochemical barrier in the plant, one presumably controlled by the shikimate pathway but not necessarily host plant-defense related. Because the root-knot nematode (*Meloidogyne javanica*) chorismate mutase, *Mj-cm-1*, suppresses soybean root vascularization, it is possible that *Hg-cm-1* is playing a similar role and the *1A* allele is more effective than the *1B* allele. Further experiments using plant material segregating for nematode resistance will be needed to show whether *Hg-cm-1A* or a closely linked gene is associated with the suppression of nematode resistance in PI88788.

Nevertheless, *Hg-cm-1* still is a strong candidate for a gene that suppresses host plant resistance to *H. glycines*. *Hg-cm-1A* and *Hg-cm-1B* are not completely selected for or against, suggesting that other genes play partial roles in *H. glycines* parasitism of PI88788. Our observations are consistent with genetic data that show that *H. glycines* contains multiple parasitism genes for reproduction on resistant plants, for example, *ror-1*, *ror-2*, *kr3*, and *kr4* (Dong and Opperman (1997). Field populations of *H. glycines* rarely show growth or nongrowth phenotypes. These populations often are sufficiently variable that they readily adapt to *H. glycines*-resistant germ plasm (Anand et al. 1995; Colgrove et al. 2002; Noel and Edwards 1996). Multiple independently segregating parasitism genes, some of which may be tandemly repeated, may explain the variable and complex pathogenic adaptability of *H. glycines*. Considering that soybean resistance to *H. glycines* is controlled by multiple resistance genes (Concibido et al. 2004), it is not surprising that nematodes may have evolved an equally

complex series of genes that allow them to parasitize resistant plants. Interestingly, the *H. glycines*-resistant plant PI88788 appears to have an unusual resistance gene (Concibido et al. 2004) and the plant cellular response to nematodes is different from that of other *H. glycines*-resistant plants, in that PI88788 resistance is marked by accumulation of cisternae and rough endoplasmic reticulum and does not show thickened cell walls, appositions, or cellular necrosis (Noel 2004).

It should be noted that we expected to see an increase in *Hg-cm-1B* in nematodes grown on PI90763 based on a correlation of the *Hg-cm-1B* with parasitism on that source of resistance (Bekal et al. 2003). The fact that we did not see this increase in *Hg-cm-1B* suggests that the selection exerted by PI90763 may not have been as great as that of PI88788, or the linkage was not as tight. Consequently, it may take many generations to gradually increase *Hg-cm-1B* allelic frequency. Long-term *Hg-cm-1* allele selection experiments are underway to test this possibility.

In summary, this population selection experiment shows that *Hg-cm-1A* increased in frequency in nematodes growing on PI88788, but not on other nematode-resistant soybean lines. This result suggests that *Hg-cm-1A* is the preferred allele for *H. glycines* parasitism of PI88788 soybean, but it is unclear whether the mechanism of selection is due to *Hg-cm-1A* suppression of host plant resistance or the manipulation of the shikimate pathway by *Hg-cm-1A* in some other way that benefits the nematode. We also showed that the *Hg-cm-1* gene family is repeated in the *H. glycines* genome. The repeated *Hg-cm-1* gene structure may provide an extra source of genetic variation to assist the nematode population in adapting to host plants or it may be a way of amplifying the expression of *Hg-cm-1* enzyme in the nematode. The application of QPCR to assess the *H. glycines* genotype was effective in monitoring allelic frequencies in individuals and in populations. This method of population genetic analysis may be useful for the study of new putative parasitism genes alleles, as they are identified, to determine whether they are selected by different host plants. The analysis of *H. glycines* parasitism of resistant soybean ultimately may lead to a method to predict *H. glycines* parasitism of soybean, which in turn will aid in the control of this damaging pathogen by allowing appropriate resistant host plants to be matched to the *H. glycines* population in the field.

MATERIALS AND METHODS

Nematode lines.

Four *H. glycines* inbred lines (TN16, TN19, TN20, and VL1) have been maintained in the greenhouse for over 60 generations. The inbred lines TN16, TN19, and TN20 were selected by single-cyst descent on resistant soybean lines PI209332, Hartwig, and PI437654, respectively. VL1 line was inbred by mass selection from an *H. glycines* population maintained on PI88788. All nematode cultures were maintained as described by Niblack and associates (1993). The HgType for TN19 and TN20 is 1, 2, 3, 4, 5, 6, 7; for TN16 is HgType 1, 2, 5, 7; and for VL1 is HgType 2, 5, 7. The number in the HgType refers to 10% or more reproduction on an *H. glycines*-resistant soybean indicator line. The soybean PI lines were 1 (PI54840), 2 (PI88788), 3 (PI90763), 4 (PI437654), 5 (PI209332), 6 (PI89772), and 7 (PI548316) (Niblack et al. 2002).

H. glycines crosses.

Three crosses, TN16 (female) $\times$ TN 20 (male), TN20 (female) $\times$ TN16 (male), and VL1 (female) $\times$ TN19 (male), were produced. Nematode and plant cultures, as well as nematode mating, were conducted in a controlled growth-chamber envi-

ronment ($27 \pm 2^\circ\text{C}$) using a modified procedure described by Dong and Opperman (1997).

To obtain female *H. glycines* parents, J2s were inoculated (1,500 J2 per plant) onto a 7-day-old soybean seedling (cv. Lee 74) growing in a 50-ml tube half filled with autoclaved sandy loam soil. Soil was washed from the roots of the soybeans 7 to 10 days after *H. glycines* inoculation and then transferred into 200-ml plastic beakers filled with hydroponic solution. The recipe for the hydroponic solution is described by Lambert and associates (1992). The hydroponically growing plants were aerated constantly by passing a stream of air through a 2-mm plastic tube connected to an aquarium pump. *H. glycines* males were removed daily from the hydroponic vessel once they started exiting the root at 10 to 14 days post inoculation. The virgin *H. glycines* females were used for mating 25 to 30 days after inoculation of the cultures, when emergence of males from those cultures had stopped completely.

To obtain *H. glycines* males for the crosses, Lee 74 soybean plants were inoculated with inbred *H. glycines* and transferred to hydroponic culture as described above approximately 1 week after cultures of female parents were established. The males were collected from the bottom of the beaker 2 weeks after inoculation.

To conduct the *H. glycines* cross, soybean plants containing virgin females were carefully transplanted into a 150-ml beaker and filled with sterile sandy soil; then, approximately 200 freshly harvested males were pipetted onto the soil surface. Hybrids nematodes (F_1) were harvested 10 to 15 days after mating by washing egg-filled cysts from the soybean roots using standard *H. glycines* extraction procedures.

To obtain the F_2 generation of nematodes, 1-week-old seedlings of Lee 74 were inoculated with the J2s hatched from the bulked F_1 eggs. The nematodes were allowed to mate randomly for one generation (30 days). The cysts were collected and the J2s hatched from the bulked F_2 eggs; then, 1-week-old seedlings of Lee 74, PI88788, PI90763, and Hartwig were inoculated with the F_2 J2s and transferred to hydroponic culture as described above.

Oligonucleotides and probes.

Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.) and the TaqMan (TM) probes with 3' end minor groove-binding nonfluorescent quenchers (MGBNFQs) were synthesized by Applied Biosystems (Foster City, CA, U.S.A.). The TM probes and primers were designed using Primer Express software (Applied Biosystems) as follows. Hg-cm-1-TM-F: 5'-CCAAGGACGTGGTCAATTACA-3', Hg-cm-1-TM-R: 5'-CCCTGCGCCGAAACACT-3', Hg-cm-1B-TM: 5'-VIC (reporter dye)-TTCATAACAACATCTCAATCG-MGBNFQ-3', Hg-cm-1A-TM: 5'-6FAM (reporter dye)-TTCA TCAAAACATCTCAATCG-MGBNFQ-3', Hg-cm-inv-F: 5'-CAATCGATGATTTTGTACGG-3', and Hg-cm-inv-R: 5'-TTGT AATTGACCACGTCCTT-3'.

QPCR genotyping of *H. glycines*.

This assay took advantage of nucleotide polymorphisms that differentiated *Hg-cm-1A* and *1B*. One minor-groove binding probe (Hg-cm-1A-TM) hybridized to *Hg-cm-1A* and was labeled with a 6-FAM dye and the other (Hg-cm-1B-TM) hybridized to *Hg-cm-1B* and was labeled with a VIC dye. These probes hybridize to the same region of DNA; therefore, the same primer pair (Hg-cm-1-TM-F and Hg-cm-1-TM-R) was used to detect both genes. The *Hg-cm* SFP assay was optimized to function as a multiplex assay to detect both *Hg-cm-1A* and *1B* simultaneously. In all assays, serial dilutions of purified plasmids containing *Hg-cm-1A* and *1B* were used as positive controls, whereas water was used as a negative control. QPCR was conducted on

an ABI PRISM 7900HT Sequence Detection System following the procedures described in the user guide (Applied Biosystems) and the procedures described by Heid and associates (1996) and Gibson and associates (1996). The following conditions were used for *H. glycines* TM assay: for each reaction, 12.5 μl of 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), 10 mM each oligonucleotide primer (Hg-cm-1-TM-F and Hg-cm-1-TM-R), and 2 μM each TaqMan probe (Hg-cm-1B-TM and Hg-cm-1A-TM) was mixed in a total volume of 20 μl . *H. glycines* DNA (5 μl) was added to a 96-well QPCR plate; then, 20 μl of the PCR reaction mix was added to complete the QPCR reaction set up. The PCR conditions consisted of 2 min at 50°C , 10 min at 95°C , then 40 cycles of 15 s at 95°C and 1 min at 62°C . The QPCR reaction was performed in absolute quantification mode and, therefore, reported copies of target DNA detected. The cycle threshold was adjusted to just above the background level in the amplification reaction and ranged from 0.2 to 0.1. A set of standards from a dilution series of *Hg-cm-1A* and *Hg-cm-1B* plasmids (Bekal et al. 2003) was used to calculate copy numbers of each allele of the *Hg-cm-1A* and *1B* genes. Five standards for each *Hg-cm-1* form were used (5 μl each standard), and each standard had 3.4×10^5 , 3.4×10^4 , 3.4×10^3 , 3.4×10^2 , or 3.4×10^1 copies.

Crude *H. glycines* lysates were prepared from J2 by transferring each individual J2 into 5 μl of sterile water on a microscope slide, then squashing it with a cover slide under a dissecting microscope. The resulting *H. glycines* extract was transferred immediately into a 96-well QPCR plate. Virgin females were squashed individually in 20 μl of sterile distilled water in 1.5-ml tubes, using a tissue grinder. Only 5 μl of the resulting extract was transferred into a 96-well QPCR plate.

Statistical analysis.

The copy numbers of the *Hg-cm-1A* and *Hg-cm-1B* alleles in the F_2 generation also were analyzed by constructing a three-way frequency table for cross per soybean cultivar per allele type. Then, stratifying the results by cross, the Cochran-Mantel-Haenszel statistic was calculated using SAS Proc Freq (Stokes et al. 1995) for soybean cultivar per allele type, to test the hypothesis of an association between allele type and soybean resistance.

PCR amplification between *Hg-cm* genes.

Using PCR primers Hg-cm-inv-F and Hg-cm-inv-R and genomic DNA (100 ng) from *H. glycines* TN19, TN20, TN16, and VL1, the PCR reaction was performed using KlenTaq polymerase, supplied buffer (Ab Peptides, St. Louis), 0.2 mM dNTP, and 10 pmol of each oligonucleotide primers under the following conditions: 94°C for 30 s; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 68°C for 4 min; followed by 68°C for 10 min. The PCR product was cloned into the pCRII-TA TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced in the Biotechnology Center at the University of Illinois.

DNA gel blotting.

H. glycines eggs were collected from greenhouse nematode cultures as described by Niblack and associates (1993). Packed 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.). Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, U.S.A.). Nematode DNA (5 μg) was digested with *EcoRI* at 37°C . For the partial digestions, 2 U of enzyme was used to digest the DNA for 10, 20, and 45 min, but 60 U of enzyme for 6 h was used to digest the *H. glycines* DNA completely. All digested DNA was separated on 1% agarose gels and transferred to nitrocellulose membranes using standard protocols (Sambrook

et al. 1989). *Hg-cm-1* cDNA was purified with a Chromaspin 100 column (Clontech, Palo Alto, CA, U.S.A.), then random prime labeled with P³²dCTP with the Prime-it II kit (Stratagene, La Jolla, CA). The probe was hybridized to the membrane and detected by standard protocols (Sambrook et al. 1989). Blots typically were exposed to X-ray film for 3 days at -80°C with one intensifying screen for the genomic DNA gel blots, but were exposed for only 3 h for the blots of the PCR reactions.

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LITERATURE CITED

- Anand, S. C., Koenning, S. R., and Sharma, S. B. 1995. Effect of temporal deployment of different sources of resistance to soybean cyst nematode. *J. Prod. Agric.* 8:119-123.
- Bekal, S., Niblack, T. L., and Lambert, K. N. 2003. A chorismate mutase from the soybean cyst nematode *Heterodera glycines* shows polymorphisms that correlate with virulence. *Mol. Plant-Microbe Interact.* 16:439-446.
- Colgrove, A. L., Smith, G. S., Wrather, J. A., Heinz, R. D., and Niblack, T. L. 2002. Lack of predictable race shift in *Heterodera glycines*-infested field plots. *Plant Dis.* 86 (10):1101-1108.
- Concibido, V. C., Diers, B. W., and Arelli, P. R. 2004. A decade of QTL mapping for cyst nematode resistance in soybean. *Crop Sci.* 44:1121-1131.
- Davis, E. L., Hussey, R. S., Baum, T. J., Bakker, J., Schots, A., Rosso, M.-N., and Abad, P. 2000. Nematode parasitism genes. *Annu. Rev. Phytopathol.* 38:365-396.
- Dewick, P. M. The biosynthesis of shikimate metabolites. 1998. *Nat. Prod. Rep.* 15:17-58.
- Dong, K., and Opperman, C. H. 1997. Genetic analysis of parasitism in the soybean cyst nematode *Heterodera glycines*. *Genetics* 146:1311-1318.
- Doyle, E. A., and Lambert, K. N. 2002. Cloning and characterization of an esophageal-gland-specific pectate lyase from the root-knot nematode *Meloidogyne javanica*. *Mol. Plant-Microbe Interact.* 15:549-556.
- Doyle, E. A., and Lambert, K. N. 2003. *Meloidogyne javanica* chorismate mutase 1 alters plant cell development. *Mol. Plant-Microbe Interact.* 16:123-131.
- Freeman, W. M., Walker, S. J., and Vrana, K. E., 1998. Quantitative RT-PCR pitfalls and potential. *Biotechniques* 26:112-125.
- Gao, B., Allen, R., Maier, T., Davis, E. L., Baum, T. J., and Hussey, R. S. 2002. Identification of putative parasitism genes expressed in the esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. *Mol. Plant-Microbe Interact.* 14:1247-1254.
- Gao, B. L., Allen, R., Maier, T., Davis, E. L., Baum, T. J., and Hussey, R. S. 2003. The parasitome of the phytonematode *Heterodera glycines*. *Molecular Plant-Microbe Interactions*. 16(8):720-726.
- Gibson, U. E. M., Heid, C. A., and Williams, P. M. 1996. A novel method for real time quantitative RT-PCR. *Genome Methods* 6:995-1001.
- Golden, A. M., Epps, J., Riggs, R. D., Duclos, L. A., Fox, J. A., and Bernard, R. L. 1970. Terminology and identity of infraspecific forms of the soybean cyst nematode (*Heterodera glycines*). *Plant Dis. Rep.* 54:544-546.
- Harris, T. S., Sandall, L. J., and Powers, T. O. 1990. Identification of single *Meloidogyne* juveniles by polymerase chain reaction amplification of mitochondrial DNA. *J. Nematol.* 22:518-524.
- Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. 1996. Real time quantitative PCR. *Genome Methods* 6:986-994.
- Huang, G. Z., Gao, B. L., Maier, T., Allen, R., Davis, E. L., Baum, T. J., and Hussey, R. S. 2003. A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode *Meloidogyne incognita*. *Mol. Plant-Microbe Interact.* 16:376-381.
- Janssen, R., Bakker, J., and Gommers, F. J. 1991. Mendelian proof for a gene-for-gene relationship between *Globodera rostochiensis* and the *H1* resistance gene from *Solanum tuberosum* ssp. *andigena* CPC 1673. *Rev. Nematol.* 14:207-212.
- Jaubert, S., Laffaire, J.-B., Abad, P., and Rosso, M.-N. 2002. A polygalacturonase of animal origin isolated from the root-knot nematode *Meloidogyne incognita*. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 522:109-112.
- Jones, J. T., Furlanetto, C., Bakker, E., Banks, B., Blok, V., Chen, Q., Phillips, M., and Prior, A. 2002. Characterization of a chorismate mutase from the potato cyst nematode *Globodera pallida*. *Mol. Plant Pathol.* 4:43-50.
- Koenning, S. R., and Barker, K. R. 1998. Survey of *Heterodera glycines* races and other plant-parasitic nematodes on soybean in North Carolina. *Suppl. J. Nematol.* 30:569-576.
- Lambert, K. N., Tedford, E. C., Caswell, E. P., and Williamson, V. M. 1992. A system for continuous production of root-knot nematode juveniles in hydroponic culture. *Phytopathology* 82:512-515.
- Lambert, K. N., Allen, K. D., and Sussex, I. M. 1999. Cloning and characterization of an esophageal-gland-specific chorismate mutase from the phytopathogenic nematode *Meloidogyne javanica*. *Mol. Plant-Microbe Interact.* 12:328-336.
- Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Genome Methods* 25:402-408.
- Neveu, C., Jaubert, S., Abad, P., and Castagnone-Sereno, P. 2003. A set of genes differentially expressed between avirulent and virulent *Meloidogyne incognita* near-isogenic lines encode secreted proteins. *Mol. Plant-Microbe Interact.* 16:1077-1084.
- Niblack, T. L. 2004. Nematode pathogens of soybean. Pages 821-851 in: *Soybeans: Improvement, Production, and Uses*, 3rd ed. H. R. Boerma and J. E. Specht, eds. American Society of Agronomy Publishers, Madison, WI, U.S.A.
- Niblack, T. L., Arelli, P. R., Noel, G. R., Opperman, C. H., Orf, J. H., Schmitt, D. P., Shannon, J. G., and Tytko, G. L. 2002. A revised classification scheme for genetically diverse populations of *Heterodera glycines*. *J. Nematol.* 34:279-288.
- Niblack, T. L., Heinz, R. D., Smith, G. S., and Donald, P. A. 1993. Distribution, density, and diversity of *Heterodera glycines* populations in Missouri. *Ann. Appl. Nematol.* 25:880-886.
- Noel, G. R. 2004. Soybean response to infection. Pages 131-151 in: *Biology and Management of Soybean Cyst Nematode*, 2nd ed. D. P. Schmitt, J. A. Wrather, and R. D. Riggs, eds. Schmitt and Associates of Marceline, Marceline, MO, U.S.A.
- Noel, G. R., and Edwards, D. I. 1996. Population development of *Heterodera glycines* and soybean yield in soybean-maize rotations following introduction into a noninfested field. *J. Nematol.* 28:335-342.
- Popeijus, M., Blok, V. C., Cardle, L., Bakker, E., Phillips, M. S., Helder, J., Smant, G., and Jones, J. T. 2000. Analysis of genes expressed in second stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* using expressed sequence tag approach. *Nematol.* 2:567-574.
- Qin, L., Kudla, U., Roze, E. H. A., Govers, A., Popeijus, H., Nieuwland, J., Overmars, H., Jones, J. T., Schots, A., Smant, G., Bakker, J., and Helder, J. 2004. A nematode expansin acting on plants. *Nature* 427:30.
- Qin, L., Overmars, H., Helder, J., Popeijus, H., van der Voort, J. R., Groenink, W., van Koert, P., Schots, A., Bakker, J., and Smant, G. 2000. An efficient cDNA-AFLP-based strategy for the identification of putative pathogenicity factors from the potato cyst nematode *Globodera rostochiensis*. *Mol. Plant-Microbe Interact.* 13:830-836.
- Riggs, R. D., and Schmitt, D. P. 1988. Complete characterization of the race scheme for *Heterodera glycines*. *J. Nematol.* 20:392-395.
- Roberts, F., Roberts, C. W., Johnson, J. J., Kyle, D. D., Krell, T., Coggins, J. R., Coombs, G. H., Milhous, W. K., Tripodi, S., Ferguson, D. J. P., Chakrabarti, D., and McLeod, R. 1998. Evidence for the shikimate pathway in apicomplexan parasites. *Nature* 393:801-805.
- Ross, J. P. 1962. Physiological strains of *Heterodera glycines*. *Plant Dis. Rep.* 46:766-769.
- Ross, J. P., and Brim, C. 1957. Resistance of soybeans to the soybean cyst nematode as determined by a double-row method. *Plant Dis. Rep.* 41:923-924.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Schmid, J., and Amrhein, N. 1995. Molecular organization of the shikimate pathway in higher plants. *Phytochemistry* 39:737-749.
- Schmitt, D. P., and Barker, K. R. 1987. Incidence of plant-parasitic nematodes in the coastal plain of North Carolina. *Plant Dis.* 72:107-110.
- Scholl, E. H., Thorne, J. L., McCarter, J. P., and Bird, D. M. 2003. Horizontally transferred genes in plant-parasitic nematodes: A high-throughput genomic approach. *Genome Biol.* 4(3):R39.

- Semblat, J. P., Rosso, M. N., Hussey, R. S., Abad, P., and Castagnone-Sereno, P. 2001. Molecular cloning of a cDNA encoding an amphid-secreted putative avirulence protein from the root-knot nematode *Meloidogyne incognita*. *Mol. Plant-Microbe Interact.* 14:72-79.
- Semple, C., and Wolfe, K. H. 1999. Gene duplication and gene conversion in the *Caenorhabditis elegans* Genome. *J. Mol. Evol.* 48:555-564.
- Smant, G., Stokkermans, J. P. W. G., Yan, Y., de Boer, J. M., Baum, T. J., Wang, X., Hussey, R. S., Gommers, F. J., Henrissat, B., Davis, E. L., Helder, J., Schots, A., and Bakker, J. 1998. Endogenous cellulases in animals: Isolation of β -1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes. *Proc. Natl. Acad. Sci. U.S.A.* 95:4906-4911.
- Stokes, M. E., David, C. S., and Koch, G. G. 1995. Categorical Data Analysis Using the SAS System. SAS Institute Inc., Cary, NC, U.S.A.
- Thomas, W. K., Vida, J. T., Frisse, L. M., Mundo, M. and Baldwin, J. G. 1997. DNA sequences from formalin-fixed nematodes—integrating molecular and morphological approaches to taxonomy *J. Nematol.* 29:250-254.
- Triantaphyllou, A. C. 1975. Genetic structure of races of *Heterodera glycines* and inheritance of ability to reproduce on resistant soybeans. *J. Nematol.* 7:356-363.
- Wang, X., Allen, R., Ding, X., Goellner, M., Maier, T., de Boer, J., Baum, T. J., Hussey, R. S., and Davis, E. L. 2001. Signal peptide selection of cDNA cloned directly from esophageal gland cells of the soybean cyst nematode *Heterodera glycines*. *Mol. Plant-Microbe Interact.* 14:536-544.
- Wrather, J. A., Anderson, T. R., Arsyad, D. M., Tan, Y., Ploper, L. D., Porta-Puglia, A., Ram, H. H., and Yorinori, J. T. 2001. Soybean disease loss estimates for the top 10 soybean-producing countries in 1998. *Can. J. Plant Pathol.* 23:115-121.