Modulations in Gene Expression and Mapping of Genes Associated with Cyst Nematode Infection of Soybean

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Infection of the soybean root by the soybean cyst nematode (SCN) (Heterodera glycines Ichinohe) induces a well-documented, yet poorly understood, response by the host plant. The plant response, involving the differentiation of a feeding structure, or “syncytium,” facilitates the feeding and reproduction of the nematode to the detriment of the host. We used a genetic system involving a single dominant soybean gene conferring susceptibility to an inbred nematode strain, VL1, to characterize the nematode-host interaction in susceptible line PI 89008. The restriction fragment length polymorphism marker pB053, shown to map to a major SCN resistance locus, cosegregates with resistance among F2 progeny from the PI 89008 × PI 88287 cross. Cytological examination of the infection process confirmed that syncytium development in this genetic system is similar to that reported by others who used noninbred nematode lines. Our study of infected root tissue in the susceptible line PI 89008 revealed a number of genes enhanced in expression. Among these are catalase, cyclin, elongation factor 1α, β-1,3-endoglucanase, hydroxymethylglutaryl coenzyme A reductase, heat shock protein 70, late embryonic abundant protein 14, and formylglycinamidine ribonucleotide synthase, all of which we have genetically positioned on the public linkage map of soybean. Formylglycinamidine ribonucleotide synthase was found to be tightly linked with a major quantitative trait locus for SCN resistance. Our observations are consistent with the hypothesis proposed by others that feeding site development involves the dramatic modulation of gene expression relative to surrounding root cells.

Additional keywords: cytology, gene mapping, Glycine max, plant-nematode interactions.

Soybean cyst nematode (SCN) (Heterodera glycines Ichinohe) infection of soybean roots presents not only an important agronomic problem to soybean growers of the midwestern and southern United States, but a fascinating phenomenon with regard to plant root cell response. The cyst nematode infection of susceptible soybean lines is accompanied by a series of cellular changes within the plant at the site of feeding initiation that leads to a structure known as a “syncytium.” Some of the defining characteristics of syncytium development include enlarged nucleoli, increased density of endoplasmic reticulum, increased number of mitochondria, changes in vacuolization, and cell wall dissolution of surrounding cells (Endo 1991). The complexity of this process suggests that the expression of several plant genes is involved in this developmental change. In fact, plant genes encoding extensin-like proteins and late embryonic abundant (LEA) protein (Van der Eycken et al. 1996), catalase (Niebel et al. 1995), cyclin (Niebel et al. 1996), membrane channel protein (Opperman et al. 1994; Wilson et al. 1994; Yamamoto et al. 1990), the E2 enzyme of the ubiquitination pathway, Myb proteins, and RNA polymerase II (Bird and Wilson 1994) have been identified to be nematode-responsive in various plant systems. Recent investigations by Hermansmeier et al. (1998) with differential displays to characterize the early compatible interaction between soybean and SCN revealed a collection of cDNAs that showed homologies to the large subunit of transcription factor TFIIA from Arabidopsis thaliana, the soybean auxin down-regulated gene ADR12, aldolase A, and the small GTP-binding soybean protein sra1, among others.

Resistance to SCN has been reported in several genotypes of soybean, and quantitative genetic analysis has allowed the development of various genetic models, generally involving multiple resistance loci acting in concert (Concibido et al. 1996b; Rao Arelli et al. 1992; Vierling et al. 1996; Webb et al. 1995). At the cytological level, early stages of the infection process in a resistant (zero to three cysts per root) line may appear quite similar to the compatible interaction (Acedo et al.
1984; Kim and Riggs 1992; Mahalingam and Skorupska 1996). Typically, these formations do not support the full life cycle of the female nematode and degenerate a few days after initiation.

The development of inbred nematode strains and their recurrent selection for zero-cyst phenotype on a resistant plant genotype has proven an effective strategy for the genetic dissection of resistance. This strategy was used in the development of an inbred nematode strain, first reported as Hg1 (Luedders 1987) and recently redesignated VL1 (Bird and Riddle 1994) with soybean resistant line PI 88287 and susceptible line PI 89008. Within this genetic system, resistance is conferred by a single recessive resistance gene (Luedders 1987). We have adopted this system to characterize the soybean–SCN interaction with emphasis on the susceptibility response by identifying a group of SCN-responsive loci in soybean. Positioning these loci on the public soybean map should allow us to identify associations, if any, to known soybean–SCN-resistance quantitative trait loci (QTL).

RESULTS

Cytological changes in roots associated with the susceptible soybean response to infection by nematode inbred strain VL1.

To confirm that observations made with susceptible host PI 89008 infected with inbred VL1 would be representative of a normal cyst nematode infection process, we conducted a cytological study during infection. Figure 1 illustrates a normal development of a feeding site. In a compatible interaction between susceptible line PI 89008 and inbred VL1, the process involved the proliferation of primary syncytium initials, radiating out with wall dissolution of adjacent cells, the formation of a highly vacuolar cytoplasm (Fig. 1B to D), and the development of cell wall in-growths typical of transfer cells (Gunning and Steer 1996) alongside xylem elements (Fig. 1D). Upon completion of the nematode life cycle, the cytoplasm of the syncytium-component cells deteriorates (Fig. 1E and F), eventually leaving near-empty cells with cell wall stubs and in-growths (Fig. 1G). In contrast, nematode penetration of the root of the resistant line PI 88287 occasionally resulted only in the formation of initial syncytium structures that degenerated before completion of a nematode life cycle, as indicated by the detachment of the cytoplasm from the cell wall (data not shown). The cytological observations resembled earlier descriptions of the resistant reaction in different genetic systems (Acedo et al. 1984; Kim and Riggs 1992; Mahalingam and Skorupska 1996).

Modulation of root gene expression in susceptible host PI 89008 in response to infection by nematode inbred strain VL1.

The dramatic cytological changes induced in the soybean root upon infection by the cyst nematode imply that several plant genes are recruited by the nematode to act in concert in order to facilitate syncytium development. Empirical selection (from literature), cDNA subtraction, and differential display produced a collection of plant genes that were further examined for response to VL1 infection in the susceptible host PI 89008. Several gene candidates were selected on the basis of previous reports (Cramer et al. 1993; Laxalt et al. 1996; Niebel et al. 1995; Niebel et al. 1996; Van der Eycken et al. 1996; Wang et al. 1995; Yoshikawa et al. 1990). Gene expression was assayed by reverse transcription of mRNA followed by polymerase chain reaction (RT-PCR). The responsive genes examined here are listed by their identity and origin in Tables 1 and 2. Selected clones that were enhanced in expression but whose identity could not be determined do not appear in the tables.

Table 1. Clones identified by differential display and cDNA subtractive hybridization

<table>
<thead>
<tr>
<th>cDNA Identity</th>
<th>Clone designation*</th>
<th>Database accession no. b</th>
<th>Oligonucleotide primers for reverse transcription polymerase chain reaction (RT-PCR)</th>
<th>RT-PCR results c</th>
<th>Mapped</th>
</tr>
</thead>
</table>
| Elongation factor EF-1α | pSMK2D             | U95397                  | 5′ > GCTGGTATCTCCAAGGATGG < 3′  
5′ > GCCTTGAGGACCCGAGTCTCGATACG < 3′  
5′ > GGATTTAAGACCCAAAATAAC < 3′  
5′ > GTGCAAATCTAGGACGACCT < 3′  
5′ > GAAGAAATCTTCTCTGCTGTCAAG < 3′  
5′ > AGACACCAAGAGAACCGYGG < 3′  
5′ > CATGCAGCCATGGCTTCGTC < 3′  
5′ > CGCATTCAGATTTGGCTATTTG < 3′  
5′ > CGCTTGATGAGGCTACGCACC < 3′  
5′ > GGGACCTGTGATTCTTCATAAT < 3′  
5′ > GCTGGTATCTCCAAGGATGG < 3′  
5′ > CGTACAGAACAAGAACTCCTCC < 3′  
5′ > ATGCACCTTTTCGTTTGTTTCGGAG < 3′  
5′ > CCTTGCTTCTTGCAGCTATCTCC < 3′  
5′ > CGCATTCAGATTTGGCTATTTG < 3′  
5′ > CGCTTGATGAGGCTACGCACC | + (weak)           |                        |
| β-glucosidase       | pSMK7D             | AF000378                | + (weak)                                                  | Control         |        |
| EC 3.2.1.21         | pSMK8S             | AF000379                | + (weak)                                                  | Control         |        |
| Heat shock protein HSP70 | pSMK251S         | AF294324                | + (weak)                                                  | Control         |        |
| Nematode 28S rRNA   | pSMK230-188S       | AF294325                | + (weak)                                                  | Control         |        |
| 30 S ribosomal protein S5 | pSMK11D          | AF294394                | + (weak)                                                  | Control         |        |
| Small ribonucleoprotein E | pSMK230-188S    | AF294325                | + (weak)                                                  | Control         |        |
| Soybean 18S rRNA    | pSMK12D            | AF061565                | + (weak)                                                  | Control         |        |
| β-tubulin           | pSMK1S             | U21296                  | + (weak)                                                  | Control         |        |
| FGAM synthase       | PSMK3D             | AF000377                | +++ Yes                                                  | Control         |        |

a Clones obtained by differential display (clone designation ending in D) and obtained from subtractive hybridization (clone designation ending in S). Among the nine clones, one was clearly of nematode origin. Whereas β-tubulin showed an identical sequence to the available database sequence, the EF-1α and HSP70 clones were divergent from the described soybean sequences within the database.

b From the GenBank, EMBL, and DDBJ databases.

c +++ = highly enhanced, ++ = moderately enhanced, and + = slightly enhanced gene expression on nematode infection of the susceptible host. = no enhanced expression. Genes that served as internal control for reaction and template concentrations are designated control.
To prepare the mRNA used to construct the cDNA subtraction library and carry out the differential display procedures, it was necessary to identify the most densely populated root tissues with feeding sites and to estimate the timing of syncytium development. Inoculations were made with prepared eggs rather than stage J2 nematode juveniles; as a result, infections were not well synchronized. The infection results, presented in Table 3, show that from day 10 the resistant and susceptible root responses are readily distinguishable. Because we were most interested in the changes of the soybean root gene expression pattern defining the accommodation of sedentary females in the root (approximately day 10 after infection) (Table 3), root material taken from PI 89008 at days 3 to 21 after infection were pooled for total RNA–mRNA isolation. For control mRNA preparation, noninfected PI 89008 root material of the same developmental stages was collected and pooled. Data in Table 3 provide only a timeline for infection phenotype under inoculation conditions used in our study and not considered a formal analysis of the infection process.
To test for enhanced expression by RT-PCR, careful dissection of the regions encompassing the feeding sites was carried out, producing extremely small tissue samples (approximately 5 mg per collection). Subsequent preparation of poly(A)^+ RNA resulted in a very low mRNA yield. Thus, a semi-quantitative assay for template concentration was developed with RT-PCR on the basis of known concentrations of leaf mRNA (Fig. 2A). RT-PCR assays were carried out with mRNA templates from infected and uninfected root of the susceptible line PI 89008 and leaf tissues. Results of the RT-PCR assay, with regard to enhanced amplification upon infection, are summarized in Tables 1 and 2, with sample RT-PCR results demonstrated in Figure 2B, results of which were independently replicated in subsequent RT-PCR assays with spectrophotometrically quantified RNA templates (data not shown). Clones that were regarded as enhanced in their expression had to show comparable differential expression with the use of both templates.

RT-PCR results show that several genes were enhanced in their expression in the VL1-induced syncytia within the roots. Many of these clones were unidentifiable on the basis of sequence analysis and database searches (data not shown), whereas other clones selected for RT-PCR could be identified on the basis of DNA sequence homologies (Tables 1 and 2). The expression of all selected genes was readily detected in leaves (except for clones of nematode origin), although some were expressed at comparable levels in infected root tissue but at undetectable levels (with our assay) in the uninfected control. Increased template concentration in the uninfected control allowed visualization of the RT-PCR products, indicating that gene expression was enhanced by nematode infection.

With the design of the primers matching divergent DNA regions, the assay discriminated between individual genes from within families such as HSP70, endoglucanase, and cyclin. Only particular genes from each class demonstrated enhanced expression in infected root (Fig. 2B).

The cytology of syncytium development and enhanced expression of several genes suggested a strong influence of the nematode on cellular processes during infection. Therefore, it was appropriate to further investigate the genetics of plant-nematode interaction in this system. First, we examined the genetics of soybean susceptibility to nematode inbred strain VL1, providing a tentative location of a gene conferring susceptibility on the public soybean map. We then determined the map locations of several of the soybean genes responsive to nematode infection.

Genetics and mapping of susceptibility
in PI 88287 to nematode inbred VL1.

Genetic characterization of the inbred nematode strain VL1 was limited to examining its ability to establish a compatible interaction on several host differentials commonly used for the assignment of infection pathotype (Golden et al. 1970). Table 4 shows the results of infection with VL1 inbred strain on host differentials, allowing us to deduce that VL1 behaves as a pathotype or “race” 5.

We used VL1 to confirm Luedders’ results (1987) regarding the presence of a single gene in PI 88287 that confers recessive resistance to cyst nematode. In the VL1 screening procedure, susceptible and resistant classes were distinct, as evidenced in Tables 3 and 5, with resistance classified as a zero to three cyst phenotype. An F1 progeny from a PI 89008 × PI 88287 cross produced a wider range of cysts per plant than did the parents under controlled growth conditions (Table 5). Despite this variation, the F1 population was susceptible to VL1.

The number of cysts per plant varied among the susceptible class in the F2 population, although the data best fit a 1:3 model (resistant–susceptible) of a single dominant gene for cyst nematode susceptibility (Table 5) and the additive gene action was not evident (data not shown). A PI 89008 × PI 88287 cross population of 55 F2 plants was used to map the susceptibility locus on the basis of linkage associations with known markers on the public soybean map. The population segregated 40:15 (susceptible–resistant) (χ^2_1 = 0.148). Restriction endonuclease digestion of genomic DNAs with BamHI enzyme allowed the detection of a dominant (45:10, χ^2_2 = 1.36) DNA polymorphism (data not shown) with marker B_053 from linkage group G as probe (Showemaker et al. 1996). The polymorphic fragment demonstrated linkage (χ^2_9:3:1 = 28.3, P < 0.01) with the susceptible phenotype in segregating F2 progeny. Of the 55 plants tested, five appeared recombinant for the marker. It was not feasible to further resolve the map position of the susceptibility locus in this mapping population because of lack of DNA polymorphisms for other markers in the vicinity (Bmgl22, C006V; 10 enzymes tested).

Linkage of the SCN resistance gene to this map loca-
tion was further confirmed by the identification of a randomly amplified polymorphic DNA marker (RAPD) linked to the susceptibility locus with bulked segregant analysis (Michelmore et al. 1991). This marker (SCN_rad) also mapped to the region encompassing marker B_053 (Fig. 3). Linkage detected with marker B_053 and SCN_rad allowed us to tentatively assign the map position of the susceptibility locus to linkage group G (Fig. 4). This is the vicinity in which a major cyst nematode resistance QTL was identified in two other studies by multiple sources of resistance (Concibido et al. 1996b; Webb et al. 1995). On the basis of these data, it is not possible to distinguish whether the resistance locus in PI 88287 is allelic to any of the loci reported previously.

Map locations of eight SCN-responsive loci in soybean.

Several QTLs have been identified in association with resistance to cyst nematode in soybean (Chang et al. 1997; Concibido et al. 1994; Concibido et al. 1996a; Concibido et al. 1996b; Concibido et al. 1997; Vierling et al. 1996; Webb et al. 1995). Therefore, it was appropriate to determine whether any of the genes we identified to be responsive to nematode infection reside within a map location associated with nematode infection. In this study, eight of the SCN-responsive loci were placed on the public soybean map (Shoemaker et al. 1996).

Restriction enzymes used to detect polymorphisms in the F₂ and recombinant inbred (RIL) population were AccI, BamHI, DraI, EcoRI, EcoRV, HaeIII, Hhal, HindIII, SspI, and TaqI. In the F₂ population derived from A81-356022 and PI 468.916 (Shoemaker et al. 1996), cDNAs were mapped to 10 linkage groups (Fig. 4). In the RIL population derived from BSR 101 and PI 437.654 (Baltazar and Mansur 1992), cDNAs mapped to five linkage groups (Fig. 4). Members of the gene family elongation factor 1α (EF-1α) and HMG CoA reductase were mapped to two of these five linkage groups (A1, L, Q, and C1).

### Table 2. Previously reported soybean genes examined for enhanced expression upon nematode infection

<table>
<thead>
<tr>
<th>cDNA Identity</th>
<th>Clone designation</th>
<th>Database accession no.</th>
<th>Oligonucleotide primers for reverse transcription polymerase chain reaction (RT-PCR)</th>
<th>RT-PCR expression</th>
<th>Mapped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase, EC 1.11.1.6</td>
<td>Z12021</td>
<td>5' &gt; TGTTGCCCATCACAACATCCAC &lt; 3'</td>
<td>+++ Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin</td>
<td>D50868</td>
<td>5' &gt; GACTATTTGCGAGCTATATTG &lt; 3'</td>
<td>++ Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin</td>
<td>X62820</td>
<td>5' &gt; GACTTTCAAAAGTAACAACCT &lt; 3'</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elongation factor EF-1α</td>
<td>X56856</td>
<td>5' &gt; CAAACTCAAAGGATGACCCT &lt; 3'</td>
<td>+ Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-1,3-endoglucanase EC 3.2.1.39</td>
<td>U41323</td>
<td>5' &gt; GATGGTGTATCAGACTCTG &lt; 3'</td>
<td>--</td>
<td></td>
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<tr>
<td>β-1,3-endoglucanase EC 3.2.1.39</td>
<td>U08405</td>
<td>5' &gt; CTTCATATTCTGACCGT &lt; 3'</td>
<td>+ Yes</td>
<td></td>
<td></td>
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<tr>
<td>β-1,4-endoglucanase EC 3.2.1.39</td>
<td>U34755</td>
<td>5' &gt; GCTAGTACATTTGACGACTAGCC &lt; 3'</td>
<td>--</td>
<td></td>
<td></td>
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<tr>
<td>β-1,4-endoglucanase EC 3.2.1.4</td>
<td>U00730</td>
<td>5' &gt; CAGGTTGGTGGTCAATGAT &lt; 3'</td>
<td>--</td>
<td></td>
<td></td>
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<tr>
<td>β-1,4-endoglucanase EC 3.2.1.4</td>
<td>U00731</td>
<td>5' &gt; GCATCGATATGGGAAACTGCAG &lt; 3'</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat shock protein HSP70</td>
<td>X62799</td>
<td>5' &gt; ACTAAAGTTGGGATTAGC &lt; 3'</td>
<td>++ Yes</td>
<td></td>
<td></td>
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<tr>
<td>Hydroxymethylglutarly CoA reductase, EC 1.1.1.88</td>
<td>pSMK8182Y10</td>
<td>5' &gt; GAGGGACGTGGGAAATCAGTT &lt; 3'</td>
<td>++ Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late embryonic abundant protein type 14 (LEA14)</td>
<td>U08108</td>
<td>5' &gt; CTATGTTGCGAGAAAGATCAGC &lt; 3'</td>
<td>++ Yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From GenBank, EMBL, and DDBJ databases.
++ = highly enhanced, ++ = moderately enhanced, and + = slightly enhanced gene expression on nematode infection of the susceptible host. = no enhanced expression.

### Table 3. Development of the VL1 nematode inbred on resistant (PI 88287) and susceptible (PI 89008) soybean lines

<table>
<thead>
<tr>
<th>Time (DAI)</th>
<th>No. soybean cyst nematode/cm of root</th>
<th>Late L4 and adult</th>
<th>No. cysts per plant</th>
<th>No. soybean cyst nematode/cm of root</th>
<th>Late L4 and adult</th>
<th>No. cysts per plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>*</td>
<td>*</td>
<td>0.0</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>*</td>
<td>*</td>
<td>2.4</td>
<td>*</td>
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</tr>
<tr>
<td>4</td>
<td>4.6</td>
<td>*</td>
<td>*</td>
<td>3.8</td>
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</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>*</td>
<td>*</td>
<td>5.5</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>10</td>
<td>11.5</td>
<td>2.7</td>
<td>0.0</td>
<td>6.7</td>
<td>3.4</td>
<td>7.7</td>
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<td>12</td>
<td>2.8</td>
<td>27.3</td>
<td>0.0</td>
<td>4.3</td>
<td>7.8</td>
<td>46.2</td>
</tr>
<tr>
<td>19</td>
<td>3.3</td>
<td>64.0</td>
<td>0.0</td>
<td>2.1</td>
<td>14.6</td>
<td>63.1</td>
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<tr>
<td>21</td>
<td>3.5</td>
<td>75.0</td>
<td>0.0</td>
<td>1.8</td>
<td>15.7</td>
<td>76.3</td>
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<tr>
<td>38</td>
<td>N/A</td>
<td>N/A</td>
<td>0.0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Data represent the average of four independent experiments. # = not applicable; N/A = not available
b DAI = days after inoculation. VL1 inoculations were 4,000 eggs per plant.

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D1, I, K, P), respectively, in the F_2 population. No polymorphisms were detected with these cDNAs in the RIL population. Two of the cDNAs, glucanase (U08405) and catalase, were mapped only in the RIL population (linkage groups J and B2, respectively). Three of the cDNAs, LEA14, cyclin, and heat-shock protein (HSP70), were mapped in both populations (linkage groups G, C2, and D1, respectively). Formylglycinamidine ribonucleotide (FGAM) synthase was mapped to linkage group G in both populations. EF-1α elongation factor was polymorphic in the RIL population with the restriction enzyme EcoRV but did not link to any known linkage group.

Four cDNAs mapped within the vicinity of previously reported SCN QTL markers. EF-1α elongation factor was positioned 5.8 centimorgans (cM) from SCN QTL marker A_023 on linkage group L (Concibido et al. 1996a) in the F_2 population. LEA14 was 30.0 cM from SCN QTL marker A_378 on linkage group G (Concibido et al. 1997) in the F_2 and RIL populations. Glucanase (U08405) was 39.6 cM from SCN QTL marker locus B_032 on linkage group J (Concibido et al. 1994; Concibido et al. 1996a; Concibido et al. 1997) in the RIL population. FGAM synthase was mapped to a < 3.0 cM interval, flanked by markers Bng_122E and B_053T at the top of linkage group G (Fig. 3). Interestingly, the major soybean SCN resistance QTL (rhg1 locus) also maps to the same interval on linkage group G (Mudge et al. 1997).

Four of the cDNAs mapped in regions known to contain resistance-gene analogs (RGAs) (Kanazin et al. 1996). EF-1α cosegregated with RGA7 on linkage group L in the F_2 population. One locus of HMG CoA reductase on linkage group P in the F_2 population was located 5.7 cM from RGA8. Glucanase (U08405) was 6.7 cM from RGA3 on linkage group J in the RIL population, and HSP70 was 10.7 cM from RGA1f on linkage group D1 in the F_2 population (data not shown).

**DISCUSSION**

Complicating factors that have slowed progress in studies of soybean–nematode interactions are the genetic heterogeneity of SCN field populations, the environmental sensitivity of the infection process, and the difficulties inherent in locating...

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**Fig. 2.** Reverse transcript polymerase chain reaction (RT-PCR) analysis of soybean gene induction during infection of susceptible line PI 89008 by nematode inbred strain VL1. **Upper gel.** Titration of root mRNA against known quantities of leaf mRNA by RT-PCR with primers specific for soybean 18S rRNA. On the right, the leaf mRNA was quantified spectrophotometrically and indicated amounts were subjected to RT-PCR with soybean 18S rRNA-specific primers. This titration series was compared with a series of 1:25 to 1:25,600 dilutions of a root mRNA preparation. There was no mRNA in the control. Size of the amplification product (in base pairs) is indicated on the left. **Lower gel,** 1.2% agarose gels after electrophoresis of PCR amplification products obtained by RT of mRNA from leaf, noninfected root, and infected root tissues (three lanes each) with specific primers, corresponding to the genes indicated at the bottom. Gluc-1 = β-1,3-endoglucanase; gluc-2 = β-1,3-endoglucanase; LEA = late embryonic abundant protein type 14; EF-1α = elongation factor 1α; HSP-1 = heat shock protein HSP70; HSP-2 = heat shock protein HSP70; n-rRNA = nematode 28S ribosomal RNA; glucosidase = β-glucosidase; 18S rRNA = 18S ribosomal RNA; HMGR = hydroxymethylglutaryl CoA reductase and formylglycinamidine ribonucleotide synthase. Primers for soybean 18S ribosomal RNA were used as internal standards to equalize the amount of mRNA from the three sources used in the assays. Nematode 28S rRNA primers served as a negative control, giving a strong signal only in the mRNA sample from roots infected by nematodes. Molecular weights (in base pairs) are provided to the left.
actively developing infection sites internal to the root and invisible at the surface. This investigation was directed toward implementing a genetically simplified system to facilitate more in-depth investigation of the soybean–nematode interaction. This system utilizes a homogeneous inbred cyst nematode population together with a highly resistant plant genotype. The PI 88287 displays an unambiguous resistant phenotype. With this system, we have initiated studies into the cellular and expressional changes that accompany the infection process.

Light microscopic examinations of the susceptible and resistant plant reactions show cellular responses similar to other described soybean–nematode systems (Endo 1991; Endo 1992; Kim and Riggs 1992). In our study, we extended the examination of the feeding site development to more than one life cycle of the nematode. The appearance of cell wall ingrowths in syncytium-component cells of the susceptible reaction resemble the appearance of transfer cells that have exhibited an enhanced capacity for uptake and passage of solutes described in other plant tissues (Gunning and Steer 1996). We could not detect such cell wall ingrowths in the resistant reaction. Absence of cell wall ingrowths may limit solutes to the syncytium in the resistant line, thus leading to premature termination of syncytium development.

Gene expression studies presented here and by other groups provide evidence that several host genes are recruited in the process of establishing a compatible interaction between the nematode and the plant. Some of these are homologous to genes with known functions, including plant genes encoding structural, housekeeping, and cell-cycle proteins. It was proposed that hydroxymethylglutaryl coenzyme A reductase (HMGR) enhanced upon nematode infection as a result of nematode dependence on the plant host for sterol biosynthesis (Cramer et al. 1993). Some enzymes might be involved in a general response to pathogens, including endo-1,3-glucanase (Yoshikawa et al. 1990) and cytosolic glyceraldehyde-3-phosphate dehydrogenase (Laxalt et al. 1996). Results of our study suggest that several plant genes responsive to the nematode encode products important to normal cellular functions. It does not appear likely, from our investigations, that many genes are induced specifically or exclusively during feeding site initiation. Likewise, this is the case of plant genes induced by Meloidogyne incognita during giant cell formation (Bird 1996). In this regard, it is reasonable to suggest that the initiation of syncytia may be directed by a dramatic change in expression levels of several genes within the syncytium that are already expressed in surrounding tissues or, as has been hypothesized, that may be important to xylem development (Bird 1996).

We used RT-PCR to confirm nematode response of genes derived from cDNA subtraction and differential display. We also confirmed the approximate map location of the dominant susceptibility gene present in soybean line PI 89008 and determined the location of several of the nematode-responsive genes.

Mapping of QTLs associated with resistance to SCN has been conducted by several laboratories (Chang et al. 1997; Concibido et al. 1994; Concibido et al. 1996a; Concibido et al. 1996b; Vierling et al. 1996; Webb et al. 1995). Most report a genomic region where a molecular marker was significantly associated with resistance, which does not allow a precise positioning of the QTL on the genomic map. The use of PCR-based and publicly available markers by Concibido et al. (1996b) and the mapping of SCN resistance as a qualitative trait, however, paved the way for the definition of QTLs.
of a major SCN resistance gene between markers B_053T and Bng_122D on linkage group G (Mudge et al. 1997). Here we tentatively assigned the locus effective in PI 88287 to this same region.

We identified a few linkages between the identified nematode-responsive genes and regions containing QTLs for resistance to SCN. Some of the mapped candidate genes were linked to resistance gene analogs, several of which have been

![Diagram](image-url)

**Fig. 4.** Eight cDNAs on the soybean public genetic linkage map. Linkage groups beginning with MS are from the F2 population of the A81 356022 x PI 468.916 cross (Shoemaker et al. 1996); BSR are from the recombinant inbred line population of the BSR 101 x PI 437.654 cross (Baltazar and Mansur 1992). If a cDNA locus was mapped to the same linkage group in both populations, the BSR linkage group was placed to the right of the corresponding MS linkage group. cDNA locus names are shown in bold and boxed and include the restriction enzymes used in genomic DNA digestion. Cyclin = accession no. D50868; EF-1α = elongation factor EF-1α; GLUC-2 = β-1,3-endoglucanase (U08405); HMGR = hydroxymethylglutaryl CoA reductase; HSP70 = heat shock protein HSP70 (accession no. X62799); LEA14 = late embryonic abundant protein type 14; FGAM = formylglycinamidine ribonucleotide synthetase. Quantitative trait loci (QTL) regions associated with soybean resistance to soybean cyst nematode are indicated by a vertical bar and SCN QTL.
mapped to regions known to encode important resistance genes in soybean (Kanazin et al. 1996; Yu et al. 1996). FGAM synthetase mapped precisely to the B_053T–Bng_122D interval on linkage group G (Fig. 3). This position corresponds to the location encoding the major SCN resistance gene (Mudge et al. 1997) as well as the resistance locus mapped in this study. The relationship, if any, of FGAM to the resistance response is still unclear. FGAM corresponds to the purL locus in *Escherichia coli* (Schendel et al. 1989) and catalyzes an important step in de novo purine biosynthesis. Not surprisingly, loci associated with purine synthesis in *Arabidopsis* spp. have demonstrated enhanced expression in rapidly dividing cells (Senecof et al. 1996), and derivatives of purine nucleotides are essential to a multitude of metabolic processes. The cDNA for this gene also has turned up in a collection of stress- and defense-related genes in the albostrains mutant of barley (Hess et al. 1998). Two genes involved in the purine synthesis pathway also were shown to be highly expressed in young and immature soybean nodules, which is consistent with their role in nitrogen fixation (Schnorr et al. 1996). Our data suggest that SCN resistance in PI 88287 is a recessive trait, implying that deficiency for an essential component of the plant–nematode interaction might account for the incompatible response. Consequently, we are currently investigating the possible influence of soybean FGAM synthase regulation in the root on the establishment of a compatible interaction with the cyst nematode.

If enhanced expression is indicative of a possible involvement in SCN resistance, we would predict that additional loci containing expression-enhanced genes also might be correlated with QTLs for SCN resistance response. A small interspecific mapping population in soybean was used in a recent study to uncover a previously unidentified QTL for SCN resistance. Interestingly, this QTL mapped within a several centimorgan interval that contains the LEA14 locus on linkage group G (B. Diers, personal communication). Consequently, the establishment of map locations for particular nematode-responsive loci in this study may assist in the future identification of additional QTLs that are important to the plant resistance response.

In this investigation, we examined the appropriateness of the PI 88287–PI 89008 strain VL1 genetic system for further in-depth investigation of SCN–soybean interaction. With regard to the infection process as well as the phenotype and genetics of susceptibility, this simplified, highly selected system appears to mirror the observations previously reported in other more complex systems. The observations made in this study should now situate us to examine the nature of gene regulation in response to nematode infection and to begin to determine the degree to which interference with this nematode-responsiveness at identified loci will influence the establishment of compatibility during the infection process.

### MATERIALS AND METHODS

#### Origin of biological materials and inoculation procedures for cytological examinations.

The nematode inbred strain VL1 was provided by T. Niblack of the University of Missouri, and seeds of soybean lines PI 88287 and PI 89008 were provided by R. Nelson of the University of Illinois. Plants were grown in glass chambers at 25°C under fluorescent light illumination with a 14-h day–10-h night schedule. Seven-day-old seedlings (four per plant) were transferred to 500 cm³ of a sterilized soil and sand mixture (1:1) and inoculated with 10 cracked cysts of nematode strain VL1. An infection cycle was completed approximately 4 to 5 weeks after inoculation (Table 1). The infection site was localized by identifying sites on the root’s surface where the infecting female protrudes. For mRNA and protein preparations and for fixation and resin embedding of infected root material, roots were inspected under the stereo microscope and the protruding female was carefully removed from the infection site. The infection sites then were carefully dissected from the root and either frozen in liquid nitrogen or submerged into fixative. Although this was an effective means of delimiting infection sites, it limited us to the dissection of middle-to-late stages of syncytium development. Sections of uninfected roots of the same age were harvested in parallel for use as controls.

#### Specimen preparation for cytology.

Plant tissues were cut into small blocks of about 1 mm and immediately fixed in phosphate buffer, pH 7.3, containing 1 mM 3-maleidobenzoic acid N-hydroxyssuccinimide ester, 0.5% Triton X-100, and 50 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid for 1 h, followed by fixation in phosphate buffer containing 3% glutaraldehyde. After dehydration in an ethanol series, embedding was performed in LR White or Quetol resins (Electron Microscopy Sciences, Fort Washington, PA, U.S.A.), according to the manufacturer’s instructions. Thick sections (1 µm) were cut with a glass knife on an Ultracut-E microtome (Reichert-Jung, Vienna, Austria). The sections were mounted on glass slides, dried on a hot plate, and stained with 0.5% methylene blue and azure II in 0.5% sodium borate. The sections were then cover slipped and viewed under a Microphot-FX light microscope system (Nikon, Tokyo, Japan).

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**Table 4. Reaction of VL1 on host differential lines**

<table>
<thead>
<tr>
<th>Host differential</th>
<th>Cysts/plant</th>
<th>Reaction</th>
<th>Cysts/plant</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lee</td>
<td>372</td>
<td>SC</td>
<td>217</td>
<td>SC</td>
</tr>
<tr>
<td>Picket</td>
<td>73</td>
<td>+</td>
<td>27</td>
<td>+</td>
</tr>
<tr>
<td>Peking</td>
<td>0</td>
<td>−</td>
<td>0</td>
<td>−</td>
</tr>
<tr>
<td>PI 88788</td>
<td>415</td>
<td>+</td>
<td>155</td>
<td>+</td>
</tr>
<tr>
<td>PI 90763</td>
<td>0</td>
<td>−</td>
<td>0</td>
<td>−</td>
</tr>
</tbody>
</table>

* + = number of females and cysts recovered was greater than or equal to 10% the number on susceptible control cultivar Lee; − = number of females and cysts recovered was less than 10% the number on susceptible control cultivar Lee. SC = susceptible control.

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**Table 5. Reaction of nematode inbred VL1 on soybean lines PI 89008, PI 88287, and F₁ and F₂ plants from the PI 89008 × PI 88287 cross**

<table>
<thead>
<tr>
<th>Population</th>
<th>Cysts/plant</th>
<th>Range</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 89008</td>
<td>19</td>
<td>132.0</td>
<td>90–201</td>
</tr>
<tr>
<td>PI 88287</td>
<td>19</td>
<td>0.5</td>
<td>0–2</td>
</tr>
<tr>
<td>F₁ (resistant)</td>
<td>10</td>
<td>152.0</td>
<td>69–294</td>
</tr>
<tr>
<td>F₂ (susceptible)</td>
<td>43</td>
<td>0–3</td>
<td></td>
</tr>
<tr>
<td>F₂ (susceptible)</td>
<td>122</td>
<td>48–375</td>
<td></td>
</tr>
</tbody>
</table>

* Expected ratio: 1 resistant to 3 susceptible. χ² = 1.07, P = 0.7
Soybean clone generation and selection.
Inoculated and un inoculated lateral root tissues were collected from inoculated and non inoculated seedlings, respectively. For infected roots, tissue was sampled from roots at 6 to 14 days after inoculation and pooled for mRNA preparation (Sam brook et al. 1989). Below is a brief summary of the procedures for cDNA subtraction and differential display with tissues from infected versus uninfected susceptible line PI 89008.

Differential display and subtractive hybridization protocol.

The protocol for differential display analysis was adapted from that of Liang et al. (1995) and Liang and Pardee (1992). Total RNA was extracted from uninfected and VL1-infected roots, as outlined in Gelvin et al. (1994). Differential display protocol bands of interest were excised and eluted from the gel. These cDNAs were reamplified with the same primer combinations (primers contained restriction endonuclease [EcoRI] sites) used in the differential display, which allowed for cloning into the pGEM-3Zf vector (Promega, Madison, WI, U.S.A.).

The cDNA subtraction protocol followed procedures described in Ausubel et al. (1988), with specifications according to Wilson et al. (1994). Briefly, RNA prepared from VL1-infected (tracer) and uninfected (driver) tissues was reverse transcribed to cDNA and ligated to EcoRI adapters. After subtraction procedures, the enriched cDNAs were ligated into the EcoRI site of the pGEM-3Zf vector.

Plasmids from the clones retrieved through the above-mentioned procedures were isolated, and the inserts were retrieved for hybridization to soybean genomic DNA by digestion with EcoRI, according to standard procedures (Sambrook et al. 1989). Clones were subjected to sequence analysis by the fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham International, Little Chalfont, Buckinghamshire, U.K.) in an ALFExpress automated sequencer (Pharmacia Biotech, Uppsala, Sweden). Clone identities were determined by blasting sequences against the databases at GenBank and SWISS-PROT. Vector sequence contamination was detected with VecScreen.

Total RNA-mRNA preparation and quantification for RT-PCR.

To isolate RNA from infected tissues, areas of PI 89008 roots with heavy SCN infestations were chosen. Total RNA was extracted from these roots by the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, U.S.A.) and subjected to DNase treatment with RNase-free DNase (Stratagene, La Jolla, CA, U.S.A.). The resulting RNA was subjected to DNase treatment with specific gene primers to ensure the absence of DNA template and quantitated in a spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.). For the RT-PCR assay, 100 ng of RNA prepared from uninfected and infected tissues was taken in RNase-free microtubes and dried in a vacuum concentrator (Labconco, Kansas City, MO, U.S.A.). Afterward, the RNA was resuspended in equal volumes of RNase-free water to achieve equal concentrations of RNA per microliter. These RNA templates were used for RT-PCR, as described below.

For mRNA isolation, total leaf RNA was isolated by the RNeasy Plant Mini Kit, followed by mRNA purification with the PolyATtract System 1000 (Promega). Root mRNA was isolated directly from uninfected and infected tissues (5 mg of starting material) of soybean-susceptible line PI 89008 with the PolyATtract System 1000. Direct isolation of mRNA from root tissue was carried out in order to minimize the loss of template. Spectrophotometric quantification was not possible with root mRNA samples as a result of the minute quantities of mRNA that were isolated. Hence, quantification was carried out by a comparative dilution series (25- to 25,000-fold) with unknown starting amounts of root mRNA and known amounts of leaf mRNA that was subjected to RT-PCR. Specific primers for soybean 18S rRNA (see Table 1) were used for PCR, taking advantage of rRNA as a regular contaminant in mRNA samples purified with Oligo(dT) to measure mRNA concentrations. This approach was adopted from the Relative RT-PCR protocol by Ambion (Austin, TX, U.S.A.). After RT-PCR (for conditions, see below), the entire reaction was run on a 1.2% agarose gel, stained with ethidium bromide, photographed, and the image saved digitally (Gel Print 2000i, BioPhotonics Corp., Dexter, MI, U.S.A.).

The image was analyzed by the GPTools computer program (BioPhotonics), which quantifies the pixel intensities of each band. According to quantification results, mRNA volumes were adjusted in the RT reaction until equal intensity of the bands produced by PCR for the samples being compared was achieved. This titration allowed us to subject nearly equal amounts of template to the first-strand cDNA reaction. This approach is similar in its experimental basis to Ambion’s Relative RT-PCR protocol, wherein the 18S rRNA signal is attenuated by adjusting the ratio of nonextendible 18S primers to normal 18S rRNA primers to achieve equal signal intensities. We utilized a dilution approach that applies the same principle in allowing us to achieve equal amplification of the control 18S rRNA sequences from the samples under consideration.

Oligonucleotide primer design for RT-PCR.

Primers were designed to produce amplification products of 250 to 450 bp by RT-PCR on the basis of sequence information. We considered an overall G+C content of 60 to 65%, ending with a mandatory G or C on both ends. The calculated Tm (at 50 mM salt concentration and 50 nM primer concentration) was approximately 62°C (Breslauer et al. 1986). For genes selected on the basis of reports of enhanced expression in other plant–pathogen interactions (Cramer et al. 1993; Laxalt et al. 1996; Niebel et al. 1995; Niebel et al. 1996; Van der Eycken et al. 1996; Wang et al. 1995; Yoshikawa et al. 1990), sequence information was retrieved from the database and corresponding primers were designed. Primers discriminating among members of the same gene family were designed according to the most divergent gene sections, chosen on the basis of a prior multiple amino acid sequence alignment by the CLUSTAL program (Higgins and Sharp 1988). For cystosolic HMG CoA reductase, no soybean sequence data were available. Therefore, conservative regions of homologous plant genes were first identified by the CLUSTAL program (Higgins and Sharp 1988). Degenerate primers were designed for PCR displaying the structure 5′ > GCHAAGTGCTKGGWARGTG < 3′ and 5′ > TAWCCCCAYTICRTTRTCRTACCA < 3′ for NAPDH, and 5′ > CCWCHGCHGTCKAAYTGGAT < 3′ and 5′ > GCWCCYTMACWCC < 3′ for HMG CoA reductase. Upon sequence verification of the
cloned genomic DNA, nondegenerate, matching primers were synthesized for use in RT-PCR experiments.

Reverse transcription of total RNA–mRNA and RT-PCR.

For first-strand cDNA synthesis, the following components were included in a 25-µl reaction: 400 µM of each deoxynucleoside triphosphate (dNTP), RT buffer (50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM MgCl₂; 0.5 mM spermidine; and 10 mM dithiothreitol), 40 µM random primer (Boehringer Mannheim, Indianapolis, IN, U.S.A.), template RNA or mRNA, and DEPC-treated water. Last, we added 2.5 units of AMV reverse transcriptase (Promega; Boehringer Mannheim, Indianapolis, IN, U.S.A.) to the reaction, and the mixture was incubated at 55°C for 30 min in a thermal cycler with a heated lid (MJ Research, Watertown, MA, U.S.A.). This first-strand cDNA template was used in a normal PCR.

A 25-µl PCR master mix contained the following components: 125 µM dNTP, Taq DNA polymerase reaction buffer (50 mM KCl; 10 mM Tris-HCl, pH 9.0; and 0.1% Triton X-100), 100 nM specific primers (for primer design, see above), 2.5 units of Taq DNA polymerase, and 2.5 µl of first-strand cDNA template from the above reaction. Reactions were performed in thin-walled tubes in a PTC100 programmable thermal cycler (MJ Research). The PCR cycling profile was 94°C for 2 min for initial denaturation followed by 35 (RNA template) to 40 (mRNA template) cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and ending with an extension at 72°C for 7 min. The entire reaction was subjected to electrophoresis in 1.2% agarose, stained with ethidium bromide, and photographed. The positive control 18S rRNA template reaction was run each time with all primer sets. Each assay was run with negative controls (water in place of mRNA), and the obtained product size was evaluated to match the expected size according to available nucleotide sequence data. A single RT reaction was used to generate a template for the PCR required to test candidate genes together with control sequences for a single experiment, a process that eliminated the variation inherent in multiple RT reactions. All PCR amplification results were confirmed a minimum of four times from templates obtained independently. The possibility of amplification from genomic DNA contamination was ruled out by treatment of RNA–mRNA templates with DNase (RNase-free). As an added precaution, primers were designed for particular genes (EF-1α and HMGR) to span intron sequences and allow amplification of different-sized products from cDNA versus genomic DNA templates (data not shown).

For preparation of gene-mapping probes, the RT-PCR products were eluted from the gel with GenElute columns (Supelco, Bellefonte, PA, U.S.A.), reamplified with the same pair of primers used in its initial amplification, and run in a preparative agarose gel. The amplified fragment was retrieved from the gel in the same way, further purified with the Wizard DNA Purification Kit (Promega), and quantified spectrophotometrically.

Plant populations for genetic analysis.

Reciprocal crosses were made between PI 89008 and PI 88287. Each cross was harvested individually, and crosses that produced two- and three-seeded pods were analyzed genetically. One seed from each of 10 pods was grown in 18-cm pots containing organic soil mix to produce the F₁ generation. The plants were grown in the greenhouse, and DNA samples from each F₁ plant were evaluated by restriction fragment length polymorphism analysis to ensure that they were hybrids. A second seed from these pods was used in nematode screening experiments.

Bulked segregant analysis.

Bulked segregant analysis (Michelmore et al. 1991), in combination with RAPD PCR analysis, was used to analyze the F₂ population. Resistant (zero to three cysts per root) and susceptible (48 cysts per root) bulks each contained an equal concentration of DNA from 16 plants. Random primers were used to screen the bulks for polymorphisms.

Gene mapping.

Two soybean populations were used to map the selected cDNAs. The first was a population of 57 F₂ individuals derived from the cross of a G. max parent, A81 356022, and a G. soja parent, PI 468.916 (Shoemaker et al. 1996). The second, a population of 100 RIL in the F₆₇ generation, was derived from a BSR 101 × PI 437.654 cross (Baltazar and Mansur 1992). PI 437.654 is resistant to all known races of SCN in the United States (Meyers and Anand 1991). Resistance to SCN in this population was mapped to linkage groups A (near the I locus), G, and M (Webb et al. 1995).

Radioactively labeled RT-PCR products were used as probes against parental DNA blots of the two populations. Parental DNA of both populations was digested with DraI, EcoRI, EcoRV, HaeIII, HindIII, and TaqI. Additionally, parental DNAs from the F₂ population were digested with AccI, AluI, BamHI, BglII, HpaII, HinfI, Rsal, and SspI. Once polymorphisms were detected, the radioactively labeled probes were hybridized against the population DNAs. In the RIL population, DNA was extracted from at least 30 seedlings per line. The DNA extraction, blotting, hybridization, and autoradiography methods used followed Keim et al. (1990).

The computer program MapMaker 3.0 (Lander et al. 1987) was used to map cDNAs in the two populations. The defaults of a LOD score of 3.0, a Haldane estimation (Haldane 1919), and a maximum recombination of 30% were used. Linkage groups were identified according to the U.S. Department of Agriculture–Agricultural Research Service public map (Shoemaker et al. 1996) and gene orders were assigned. The “compare,” “try,” and “ripple” commands were used to include a locus on a preexisting linkage group.

Nematode pathotype testing.

To characterize the pattern of VL1 infection, a replicated standard race test was performed (Golden et al. 1970). For each test, three plants of differential host lines Lee, Picket, Peking, PI 88788, and PI 90763 were inoculated. SCN differential host lines were provided by the University of Illinois germ plasm bank and J. Wilcox of Purdue University.

Assay for soybean reaction to VL1 inoculation in segregating populations.

Nematode cysts were collected from the root and soil by the procedure described by Faghihi et al. (1986). Collected and rinsed cysts were crushed with a rubber stopper and passed through a 100-mesh (150 µM) sieve nested in a 400-mesh (38 µM) sieve. Intact cysts were collected on the 100-mesh
sieve and the crushing was repeated. The inoculum was dis- luted to 4,500 eggs per milliliter.

Seven F1 plants (the second seed from two- or three-seeded pods) and 165 F2 seeds were screened with VL1. Seeds were germinated in sand, and roots of 7-day-old seedlings were inoculated with 4,500 VL1 eggs per plant. Plants were grown in 7 × 10-cm pots containing a 3:1 sand–soil mixture. Pots were inoculated with 4,500 VL1 eggs per plant. Plants were grown germinated in sand, and roots of 7-day-old seedlings were inoculated with 4,500 VL1 eggs per plant. Plants were grown in a growth chamber with a 14-h photoperiod and were fertilized weekly with Masterblend 20:10:20.

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