Theor Appl Genet (2012) 124:1027–1039
DOI 10.1007/s00122-011-1766-6

ORIGINAL PAPER

Recombination suppression at the dominant *Rhg1/Rfs2* locus underlying soybean resistance to the cyst nematode

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Received: 21 December 2010 / Accepted: 4 December 2011 / Published online: 27 December 2011

Abstract Host resistance to "yellow dwarf" or "moonlight" disease cause by any population (Hg type) of *Heterodera glycines* I., the soybean cyst nematode (SCN), requires a functional allele at *rhg1*. The host resistance encoded appears to mimic an apoptotic response in the giant cells formed at the nematode feeding site about 24–48 h after nematode feeding commences. Little is known about how the host response to infection is mediated but a linked set of 3 genes has been identified within the *rhg1* locus. This study aimed to identify the role of the genes within the locus that includes a receptor-like kinase (RLK), a laccase and an ion antiporter. Used were near isogeneic lines (NILs) that contrasted at their *rhg1* alleles, gene-based markers, and a new Hg type 0 and new recombination events. A syntenic gene cluster on Lg B1 was found. The effectiveness of SNP probes from the RLK for distinguishing homolog sequence variants on LgB1 from alleles at the *rhg1* locus on LgG was shown. The resistant allele of the *rhg1* locus was shown to be dominant in NILs. None of the recombination events were within the cluster of the three candidate genes. Finally, *rhg1* was shown to reduce the plant root development. A model for *rhg1* as a dominant multi-gene resistance locus based on the developmental control was inferred.

Introduction

Soybean (*Glycine max* L. Merr.) seed yield losses due to root infestation by *Heterodera glycines* I. soybean cyst nematode (SCN) have been severe (Wrather et al. 2001). Losses have occurred since the crop was domesticated.

Communicated by I. Rajcan.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-011-1766-6) contains supplementary material, which is available to authorized users.

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SCN has become the world's most widespread and damaging soybean pathogen. Soybean resistance to SCN was found in 112 genotypes, about 1% of pre-domesticated and early-domesticated plant introductions (PI; Arelli 1994; Arelli et al. 2000). The development of partially resistant cultivars by gene introgression and other disease management measures have limited the costs of SCN infestation to the US soybean producers to about $1 billion/year in seed yield losses.

Soybean cyst nematode, like many plant parasitic nematodes, lives as an obligate endoparasite of plant roots that can use many alternate hosts (Hussey and Grundler 1998; Keen and Roberts 1998). Cultivars with different resistance genes were used to define races (Caldwell et al. 1960). However, races were found to be mixtures of biotypes (Hg types; Niblack et al. 2003). Over the past 50 years in the USA, the number of Hg types of SCN that was recognized has expanded from 4 to over 20 of a possible 1,024. Directed breeding for cultivar resistance from new sources also tends to select for new Hg types. Consequently, new resistance genes and new alleles of existing genes will be continually needed. Resistant cultivars have a Female Index (FI) of less than 10% of the cyst numbers on susceptible cultivars in parallel tests (Arelli 1994). Though arbitrary, the 10% measure of resistance often approximates to the economic loss threshold in the US soils (about 25 cyst/100 cm^3 of soil). Genetic diversity is found both in the field and in the commonly used inbred cyst populations like PA3, Hg type 0 (Arelli 1994). PA3 was derived from a field population of race 3 by incomplete inbreeding of the progeny of a single cyst (sibling mating). Therefore, recombination due to sexual reproduction, transposon-derived genome plasticity and/or mutation may continue to generate diversity in this population (Bekal et al. 2003).

Variation among the host plant roots in response to SCN has been associated with light, temperature and genetic purity of both the host and pathogen. Consequently, both genetic identities and environmental conditions must be measured and rigorously controlled during the host pathogen assays (Arelli 1994). In both resistant and susceptible cultivars, the interaction between the nematode and soybean root passed through several discernable phases (Mahalingam and Skorupska 1996; Davis et al. 2004; Gao et al. 2004). However, resistance or susceptibility of the soybean was not induced until females establish a feeding site. The feeding site developed to provide a giant cell with its own secondary root-type vasculature provides nutrients to the female cyst. Cell to cell contact occured at the syncytia through a stylet sufficiently narrow to prevent the passage of proteins and other molecules greater than 20 kDa (Davis et al. 2004). Membrane to membrane contact was inferred. Secretions from several glands of SCN contain plant growth regulators and other bioactive factors.

Inheritance of resistance to SCN was first reported in the 'Peking' PI, and three recessive gene symbols (*rhg1–rhg3*) were assigned to the underlying loci (Caldwell et al. 1960). One locus, *rhg1*, provides the major portion of resistance to SCN Hg type 0 (race 3) and Hg type 1.3.5.6.7.8 (race 14) across many genotypes whether they were derived from Peking, PI437654, PI88788, PI209332 or PI90763 (Concibido et al. 2004). However, since the co-evolution of the soybean nematode interaction was complex (Hussey and Grundler 1998), it appeared likely that the locus contained several genes with each contributing partly to the activity of the locus (Ruben et al. 2006).

The cytological studies suggest that the *rhg1* driven Peking-type resistances share mechanisms of giant cell breakdown (pronounced necrosis and cell wall appositions) not seen in PI88788 type resistances in response to Hg type (Mahalingam and Skorupska 1996). The differences in the mechanism of giant cell breakdown in Peking and PI88788 may derive from distinct alleles at *rhg1* and/or other defense-associated loci (Arelli 1994; Brucker et al. 2005). The *rhg1* locus was repeatedly located to a sub-telomeric region of the soybean molecular linkage group G by many studies (Webb et al. 1995; Chang et al. 1997; Concibido et al. 1997; Cregan et al. 1999; Meksem et al. 2001a, b). A functional *rhg1* locus on linkage group G was common to many mapped resistance sources (Concibido et al. 2004). However, some mapped resistance sources have an *rhg1*-like locus (required for resistance to all races) at another location in SCN resistant PIs (Lg B1,Vierling et al. 1996; mid LgG, Wang et al. 2001; LgB2, Yue et al. 2001), suggesting that functional paralogs of *rhg1* exist among the duplicated regions of the soybean genome (Shultz et al. 2006).

Genes underlying resistance to Hg type 0 (PA3, race 3) have been mapped with greatest accuracy using recombinant inbred lines (RILs) and near isogenic lines (NILs) derived from the cross of 'Essex' by 'Forrest' (E × F; Chang et al. 1997; Meksem et al. 2001c; Lightfoot 2008). Forrest provided a unique set of tools for genomics (Meksem et al. 2000; Wu et al. 2004; Shultz et al. 2006; Lightfoot 2008). Forrest had introgressed only resistance to Hg type 0 (Hartwig and Epps 1973) but Peking also resisted two other Hg types. Only *rhg1* and *Rhg4* were introgressed into Forrest (Meksem et al. 2001a). Several NIL populations segregating for *rhg1* and/or *Rhg4* were developed from the cross of E × F (Njiti et al. 1998; Meksem et al. 1999; Triwitayakorn et al. 2005; Ruben et al. 2006). Genomic analysis identified six genes and seven intergeneic regions within the 42 kbp identified as the locus (Ruben et al. 2006) with many differences compared to susceptible genotypes 'A3244' (Hauge et al. 2006) and 'Williams 82' (http://www.phytozome.net; Schmutz et al. 2010).

The actions of the *rhg1* resistance alleles have complex effects. First, *rhg1* alone among the resistance loci was necessary, but not sufficient, for resistance to all tested Hg biotypes (Concibido et al. 2004). Second, some *rhg1* alleles appeared to restrict seed germination (Kopisch-Obuch et al. 2005; Brucker et al. 2005). Third, in progeny with a resistance allele at *rhg1* the allele of an interacting locus on LgM was always inherited from the resistant parent (Webb et al. 1995). It was inferred from the pattern of epistasis that *rhg1* caused gamete or zygote abortion if this modifier locus was inherited in the wrong phase. Fourth, some *rhg1* alleles inhibited seed yield at harvest in the absence of SCN (Mudge et al. 2005; Yuan et al. 2002; Kassem et al. 2006). Therefore, multiple gene or locus interactions were inferred that both underlie resistance and also alter plant development (Ruben et al. 2006).

The three genes within the markers bounding the *rhg1* locus in Forrest included a receptor-like kinase (RLK; EC 2.7.11.1), a laccase (EC 1.10.3.2) and a predicted sodium/hydrogen antiporter (Ruben et al. 2006). Immediately outside the locus (on the basis of one recombination event) were two predicted proteins of unknown function. Only the RLK and laccase were present in EST collections derived from roots by 2011. In combination these three genes and their intergeneic regions provided resistance to SCN. Here was reported a syntenic gene cluster; a set of probes that distinguished among alleles and homeolog sequence variants of the RLK; an analysis of gene action and root development in NILs; screens for new recombination events; and analysis of transcripts and protein abundances encoded by the RLK *rhg1* candidate genes.

Materials and methods

Plant materials

Many of the genetic materials were described in Triwitayakorn et al. (2005) and Ruben et al. (2006). Briefly, the seeds of RILs and the NIL populations derived from the cross of E × F were obtained from Dr. Paul Gibson at Southern Illinois University at Carbondale in 1995 and were increased from 1995 to present at the Agronomy Research Center (Lightfoot et al. 2005).

NIL populations were developed and maintained as described in Triwitayakorn et al. (2005). Genetic identity and purity were checked after increase and before each experiment with 5–10 SSR markers/line and DNA from 5–10 seeds/line. All lines are available on request as seed. Seeds of NIL 34-23 (resistant haplotype between markers Satt 214 to Satt 570) and NIL 34-3 (susceptible haplotype from the most telomeric marker Satt 214 to the Sat122-Satt 570 interval) were obtained at the F5:7:13 generation (NILs had been subject to single seed descent twice, at the F5 and F7). Genotypes were *rhg1rhg1Rhg4Rhg4* for NIL 34-3 and *Rhg1Rhg1Rhg4Rhg4* for NIL 34-23 whereas NIL34-33 contained both those and *Rhg1rhg1Rhg4rhg4* in different plants.

To screen the *rhg1* locus for internal recombination events several populations were used. The ExF RIL population ($n = 100$; Meksem et al. 2001a, b, c; Lightfoot et al. 2005) was the basis of three NIL populations. NILs were collected from three populations; E × F 34 ($n = 2{,}000$) derived from heterozygous plants; E × F 11 derived from a cross of two NILs with contrasting alleles at *rhg1* ($n = 2{,}000$; Triwitayakorn et al. 2005; Ruben et al. 2006); and individual NIL plants of the heterozygous line E × F 34-33 ($n = 200$). Additional RILs from the 'Resnik' by 'Hartwig' (R × H) and the 'Flyer' by Hartwig (F × H) populations ($n = 975$; Prabhu et al. 1999) were used. Finally, the set of SCN resistant lines among available PIs ($n = 112$; Ruben et al. 2006) was used.

Near isogenic line populations and lines

Seed of soybean populations and individual lines within populations were obtained from the seed store at SIUC managed by Dr. Lightfoot. Seed of line NIL 34-33 (polymorphic haplotype between markers Satt 214 to Satt 570) was obtained at the F5:13 generation. The three genotypes found within NIL 34-33 were; *rhg1Rhg1Rhg4Rhg4; rhg1rhg1Rhg4Rhg4* and *Rhg1Rhg1Rhg4Rhg4*. Two hundred plants were screened for resistance to SCN and for new recombination events using probes described in the text.

SCN inoculations

Soybean plants were grown in 5 l buckets, each containing 20 cones in a randomized setup. Each bucket contained a 1:1 ratio of sand soil mix. The containers were placed in a water bath set at 26°C in the SIUC greenhouse. Growth conditions were a 14 h light cycle, day time temperature of 30°C and a nighttime temperature of 22°C. Infection with Hg type 0 SCN populations consisted of inoculating 2,000 eggs to each 4-day-old seedling. Inoculated soybean plants were removed from the cones; 30 days postinoculation and cyst numbers counted. Experiments were repeated.

Some experiments used a growth chamber for assays of SCN and root growth. The conditions varied from the greenhouse as follows. The whole chamber was set at 26°C. The humidity was maintained at approximately 40–50% judged by indicator cards.

The NIL experiments used single-plant replications. The cultivars 'Lee 74', 'Essex' and 'Hutcheson' were used as susceptible controls (Niblack et al. 2003). The differentials or indicator lines and the associated FI were 'PI54840' (FI 7%), PI 88788 (FI 2%), PI90763 (FI 1%), PI437654

(FI 0%), 'PI 209332' (FI 1%), 'PI89772' (FI 2%) 'PI548316' (FI 8%) and 'PI548402' (FI 3%). Therefore the standard differentials showed this HG type to be 0 (Niblack et al. 2003) corresponding to race 3 (Riggs and Schmitt 1988).

DNA and RNA extraction

DNA was isolated following a modified protocol by Saghai-Maroof et al. (1984) with the modifications noted below. Briefly, 100 mg of frozen plant tissue was ground, 600 µl preheated (65°C) extraction buffer [Tris HCl (pH 8.0) (100 mM), EDTA (pH 8.0) (20 mM), NaCl (1.4 M), CTAB (2%), 2-mercaptoethanol (0.4%)] was added, incubated at 65°C for 1 h, cooled and centrifuged at 10,000*g* for 15 min. The supernatant was decanted and 5 µl of RNase (5 mg/ml) was added at 37°C for 1 h. The aqueous phase was extracted first by the addition of equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) v/v, incubated for 2 min by slow inversion followed by centrifugation at 10,000g for 15 min and secondly by the addition of equal volume of chloroform: Isoamyl alcohol (24:1). DNA was collected by the addition of iso-propanol to the supernatant, followed by centrifugation at 12,000*g* for 5 min. The DNA pellets were washed twice with 70% (v/v) ethanol, dried and finally dissolved in 30 µl Tris buffer. Concentration of DNA was calculated by measuring absorbance at 260 and 280 nm.

Total RNA was isolated with Trizol (Invitrogen Cat. No. 15596-026, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA pellet was dissolved in 40 µl of DEPC-treated MQ water and quantified by measuring absorbance at 260 nm. First strand cDNA was synthesis carried out using oligo dT primers using a cDNA synthesis kit, according to manufacturer (Invitrogen). Presence of the *rhg1* mRNA was confirmed by PCR analysis using a *rhg1* intron flanking primers pair: *rhg1*-int-F-LRR (ATT TGA ATC AGA AGT CAG TGT) and *rhg1*-int-R-LRR (TCT GGT CTA ATC TCT TCC AGC).

NIL genotyping by markers linked to *rhg1*

About 50 ng of DNA was used for microsatellite analysis on PAGE according to Yuan et al. (2002). Amplification reactions for analysis on agarose gels were performed after Shultz et al. (2007). Primer sequences are provided in Supplementary Table 1. The markers from were used to genotype resistant and susceptible segregants from NIL 34-33 (Ruben et al. 2006).

Taqman assays of alleles the RLK within the rhg1 locus

The SNP genotyping assay within the gene encoding the RLK was performed using a custom Taqman™ Kit. Three probes were designed at base pairs 1,486, 506 and 2,040 to distinguish the 8 common alleles of the RLK (Supplemental Table 2). Only probe 1,486 was polymorphic in Essex, Forrest and the derived NILs. A 242 bp amplification reaction was carried out using an *rhg1* LRR forward primer: 5′ CAG AGA ACA ACC TCC TTG 3′ and an *rhg1* LRR reverse primer: 5′ CAG AAC CTG AGA GGC TAT 3′; IDT DNA, Coralville, IA, USA) with the following discriminatory probe pair. Probe 1: 5′-Fam-TAT TCC TTC AAG CAT TGC AAA CAT TTC CTC G-BHQ1-3′ and Probe 2: 5′ Hex -TAT TCC TTC AAG TAT TGC AAA CAT TTC CTC GC-BHQ1-3′. Primer and probe optimization were done by using different combinations of each pair and optimizing to optimal signal strength and balanced fluorophore intensity. The PCR reaction was carried out using a three step PCR protocol with one hold at 95°C for 10 min followed by 35 cycles that included a denaturation cycle of 95°C for 30 s, annealing at 58°C for 10 s and an extension at 68°C for 20 s.

SNP assays

Primers for SNPs within the *rhg1* locus on Lg G were used in fine melt curve assays using the ABI7900 with HTM software as described previously (Yuan et al. 2008) with the following modifications. Briefly, genomic DNA was used; multiple amplicon sizes were detected on PAGE gels; melt curve data were normalized by both local (local background value was subtracted from the intensity value of sample) and global metrics. Three new primer used was SIUC-SNP_A/G at 56,320 bp in the sequence of BAC B73P06 between the laccase and the gene predicted to encode a Na/H transporter.

Southern hybridization

Southern hybridizations were performed following the standard procedure described in Meksem et al. (1999). Total genomic DNA was digested with restriction enzymes, separated by electrophoresis on a 0.8% (w/v) agarose gel and transferred onto a positively charged nylon membrane. After hybridization, the corresponding bands were visualized by exposure of X-ray film for 24–48 h.

Total root protein extraction

Protein from root material was isolated from infested and non-infested roots of Forrest and Essex according to (Hurkman and Tanaka 1986; Hajduch et al. 2005). The 2 g of the finally ground powder was resuspended in 5 mL of Tris buffered phenol (pH 8.8) and 5 mL of extraction buffer (1% (w/v) SDS, 0.1 M Tris–HCl pH 8.8, 0.4% (v/v) β-mercaptoethanol, 10 mM EDTA and 0.9 M sucrose). The solution was vortexed vigorously and centrifuged at 5,000*g* for 15 min. After removal of the top phase (phe-

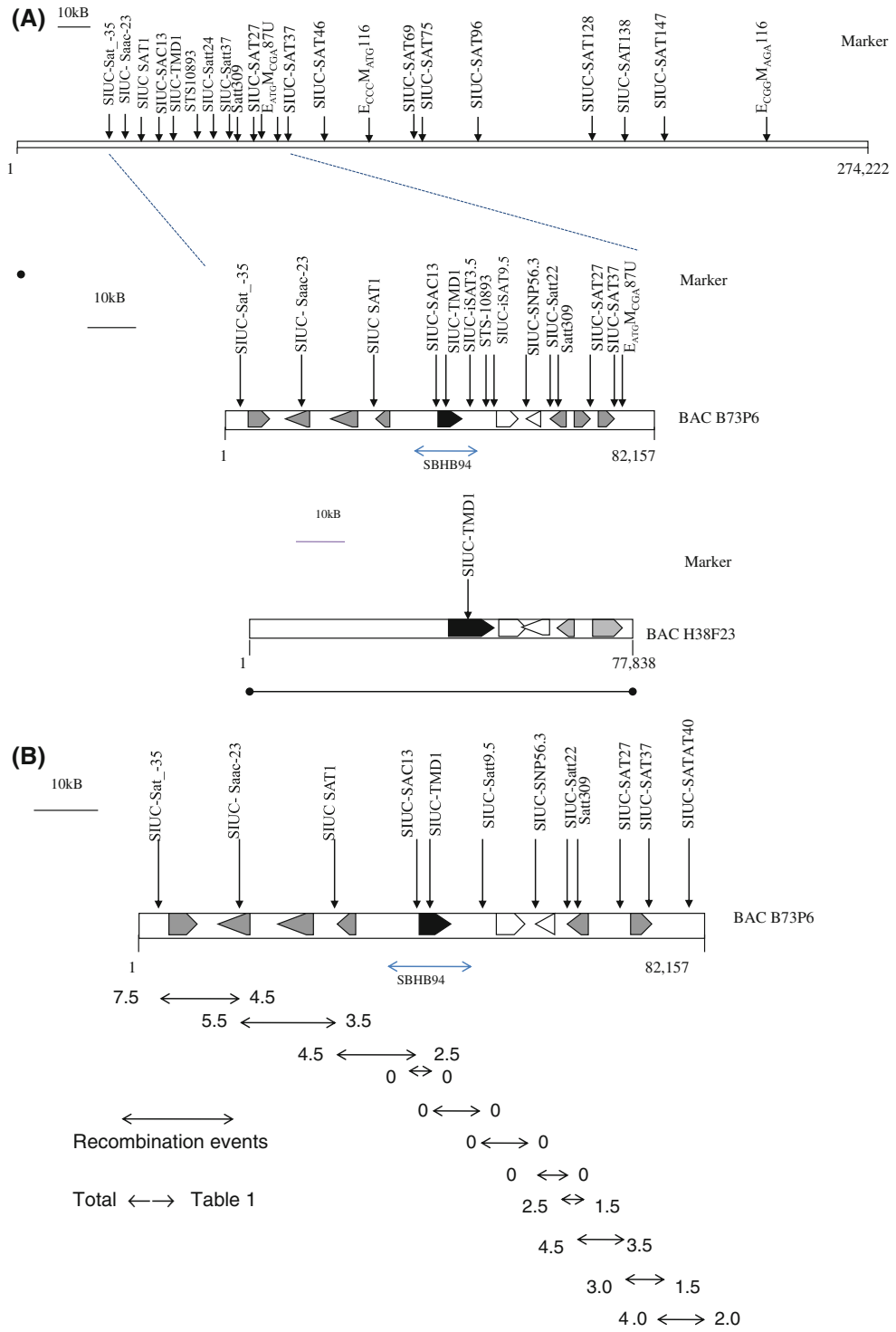

Fig. 1 Marker map of the genomic region around *rhg1*. **a** Shows a detailed locus ideogram. The marker TMD1 was within the intron of the gene encoding the RLK and amplified a different fragment from each of two paralogs (Fig. 3) of 303 ± 15 and 362 bp. Sequence coordinates were from Ruben et al. (2006) for the region and from the sequence of Forrest BAC 73p06 for the *inset*. **b** Shows the rates of recombination observed in the regions for NIL populations in *parentheses* (total NILs analyzed/NILs from Table 2B)

nol) the bottom phase was back extracted with Tris buffered phenol (5 ml) and an equal volume of the extraction buffer. Proteins were pelleted by centrifugation at 20,000*g* for 20 min and washed according to (Hajduch et al. 2005). The pellet was dried and resuspended in SDS loading buffer. Total protein concentration was

Table 1 Genes predicted from DNA sequence within the *rhg1* locus encoded by BAC 73p06 (81,157 bp) from Forrest (resistant) and sequence contig from A3244 (susceptible, AX196295)

Gene #	Annotation	Forrest	A3244	EST
Marker	SIUC_Sat_-35 (35 bp)	1,770	11,870	na
1 (5)	**NADP redox coenzyme-like**	**5,959-7,947**	**16,234-18,215**	**AW185583**
2 (6)	**Predicted protein**	**14,800-10,888**	**25,760-21,294**	**BG550903**
Marker	*SIUC_Scaa-23 (19 bp)*	*13,800*	*23,900*	*na*
3 (7)	**Predicted protein**	**23,795-18,782**	**34,265-28,983**	**TC63131**
Marker	SIUC_Sat_001 (2 bp)	24,500	34,600	na
4 (8)	**Predicted protein**	**30,076-28,429**	**40,482-38,327**	**na**
Marker	Minisatt 1 (45 bp)	33,900	44,000	na
Marker	*SIUC_Sca_005/Sac_013 (4 bp)*	*36,400*	*46,700*	*na*
5 (9)	**Receptor-like kinase (*rhg1*)**	**36,448-39,204**	**46,891-49,573**	**AF506517***
Marker	*SIUC-SattTMD1 (19 bp)*	*38,500*	*48,500*	*na*
Marker	SIUC_Satt3.5 (22 bp)	40,150	50,000	na
Marker	STS 10893 (12 bp)	43,800	54,000	GF097715
Marker	SIUC-Satt9.0 (17 bp)	45,250	54,750	na
Marker	SIUC-Satt9.5 (21 bp)	45,500	55,000	na
6 (10)	**Variant diphenol oxidase**	**47,930-52,465**	**58,247-62,782**	**AY113187***
Marker	SIUC-SNP_A/G	56,320	66,637	na
7 (11)	**Na/H antiporter-like 1**	**57,661-55,772**	**67,540-64,896**	**na**
Marker	SIUC-Satt22/Indel22 (20 bp)	58,500	58,500	na
Marker	*BARC-Satt309 (17 bp)*	*61,100*	*71,400*	*na*
8 (12)	**Na/H antiporter-like 2**	**64,165-60,904**	**74,602-69,934**	**AW279576***
9 (13)	**DNA helicase-like 1**	**64,996-73,760**	**75,245-75,418**	**na**
Marker	*SIUC-Sat_027 (3 bp)*	*65,570*	*75,881*	*na*
10 (14)	**DNA helicase-like 2**	**70,601-74,056**	**80,601-84,056**	**BF425110**
Marker	SIUC- ATG4 (5 bp)	74,150	84,400	na
Marker	SIUC- Sat_37 (4 bp)	74,250	84,500	na
Marker	Minisat 2/SIUC-Sctt39 (45 bp)	75,800	85,900	na
Marker	SIUC-Sat_40 (38 bp)	79,500	89,600	na

The gene numbers in the larger sequenced interval; the sequence coordinates from the predicted 5′ translation start to 3′ translation stop and the presence of ESTs in Genbank (May 2011) with homology to the predicted genes are shown. Marker coordinates are *listed*. Intrageneic markers were *italicized*. Genes are *bold face*. Marker positions are rounded to nearest 10 bp except for SNP markers which were exact. The *asterisk* indicates the aligned EST and mRNA are from soybean, *Glycine max*. The plasmid pSBHB94 encompassed from 30,423-40-194 bp

determined using a non-interfering protein assay (Sheffield et al. 2006).

SDS-PAGE and Western hybridization

SDS-PAGE of total plant proteins from Essex and Forrest followed by Western hybridization was carried out according to (Afzal 2007) with the following modifications. For the Westerns, a custom-made antibody generated against a peptide CTL SRL KTL DIS NNA LNG NLP ATL SNL S from the LRR domain of RHG1 was used (Alpha diagnostics, San Antonio, Texas). As a secondary antibody, an anti rabbit IgG HRP was used (GE healthcare, Milwaukee, Wisconsin).

Results

The soybean genome is hypothesized to be a diploidized tetraploid. Therefore a detailed molecular analysis of the *rhg1* locus required that paralogs and syntenic gene clusters be identified. Probes developed from the sequence of BAC 73P06 (Fig. 1; Table 1) were used.

Syntenic paralogs of *rhg1 locus*

To identify *rhg1* paralogs, primers specific to the conserved regions of *rhg1* leucine-rich repeat and *rhg1*- kinase domain were designed. The conserved regions were determined by aligning DNA sequences from known *rhg1*-like

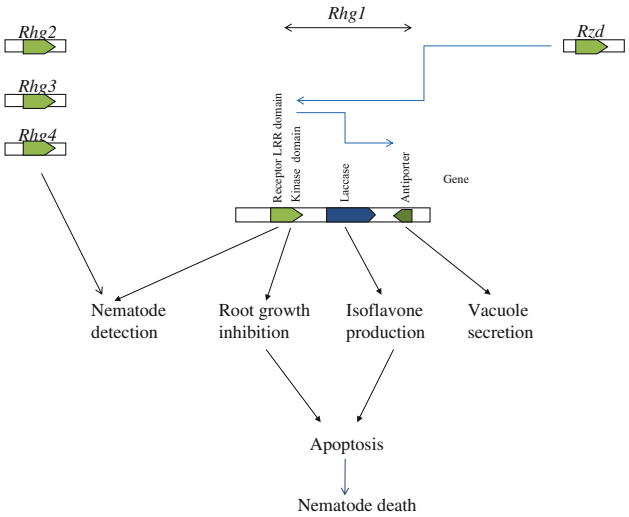

Fig. 2 Model for the function of the *rhg1* locus in resistance to SCN. *Black arrows* show positive interactions, *blue arrows* show inhibitions. In this model four primary phenotypic events are controlled by the three genes at *rhg1* and four unlinked genes *Rhg2-4* and *Rzd1* (*sup-Rhg1*). The apoptosis cause in the giant cell of the nematodes feeding sites will occur in plant root cells in the absence of *Rzd1* allele in the coupled phase with the RLK. Root growth inhibition occurs despite the suppression of the RLK

Fig. 4 The RHG1 protein alters root development. **a** Soybean NILS at 2 weeks pre-SCN inoculation show different root morphologies, postinoculation root masses are not different (by 6 weeks). Therefore, *rhg1* inhibits germination and early root growth. **b** The root morphologies cosegregated with the allele in the RLK at the rhg1 locus as shown by the intragenic marker TMD1 (satellite in the intron)

genes in different soybean cultivars and other plant species. PCR-amplified products were radiolabeled and used as probes against the Forrest MTP library. The hybridizing BAC colonies were confirmed by Southern hybridizations to purified DNA. There were five positive clones for the LRR probe and three were also positive from the kinase hybridizations (Fig. 2). One of the identified clones (B21d09) contained the *rhg1*, found on scaffold 121 whereas the other three clones contained the RLK paralogs (B10a18, B55i16 and H38f23). B10a18 and B55I16 were on Lg A1 scaffold 15; H38f23 was on Lg B1 and scaffold 139 (69100-144000). Significantly, this BAC and scaffold 139 contained a complete set of syntenic genes for a second *rhg1* locus (the RLK, laccase both antiporters, the kinase and the helicase; Supplemental Fig. 1). The DNA markers sequence paralogs were present but more diverged. Sequence analysis of the alleles in Williams 82 and Forrest the RLK at *rhg1* showed 99% amino sequence identity. Among the paralogs DNA sequence identity was high (~92% in geneic region; Supplemental Fig. 1). Amino acid identity was 84% in the LRR, 86% in the transmembrane domain and 94% in the kinase domain. The laccase and the antiporter also showed 85–96% amino acid identity.

Both of the RLK paralogs were located by BLAT of BES to sequence scaffolds and were in regions where loci with functions similar to *rhg1* were located Lg A1 (the RLK; Vierling et al. 1996) and LG B1 (the syntenic cluster; Wang et al. 2001). The paralogs may encode proteins that recognize novel race biotypes or substitute for *rhg1* following activation in certain PIs or crosses.

Allele discrimination

Since homeolog sequence variants appeared to exist for each gene in the cluster it was important to distinguish alleles precisely and separately from homolog sequence

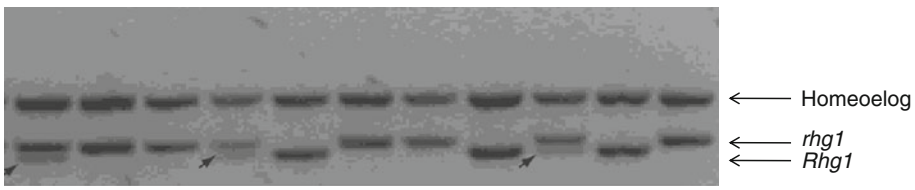

Fig. 3 Analysis of NIL 34-33 with TMD1. Out of the 34 plants analyzed, 4 were heterozygous (*lanes 12*, *14*, *20* and *29*). The four heterozygous lines had a resistant phenotype. SCN counts and gel scores for the plants were given in Table 3

Table 2 DNA marker segregation within all the SCN resistant NILs and seven selected susceptible NILs from the ExF34 derived NIL population (genotypes 1–40)

Lg	Mean	A2	G	G	GorM?
			4.5 cM	12.7 cM	52.9 cM
	Female index	BARC-BLT65	BARC-Satt309	BARC-Satt570	BARC-Satt594
A. RILs					
RIL62	0.00	B	B	A	B
RIL64	0.17	A	A	B	A
RIL84	0.59	B	A	A	B
RIL61	0.76	A	B	B	B
RIL26	1.01	B	B	B	B
RIL32	1.01	B	B	A	B
RIL89	1.01	B	B	B	B
RIL37	1.09	B	B	B	B
RIL58	1.26	B	B	B	B
RIL67	1.26	B	B	B	B
RIL17	1.34	B	B	A	B
RIL5	1.43	B	B	B	B
RIL78	1.43	B	B	B	B
RIL59	1.93	B	B	B	B
RIL21	2.10	B	B	B	B
RIL57	2.10	A	B	B	B
RIL11	2.27	B	H	H	A
RIL65	2.44	B	B	B	B
RIL74	2.44	A	B	B	B
RIL47	2.61	B	B	B	B
RIL23	3.70	B	B	B	B
RIL3	8.82	B	B	B	B
RIL6	13.11	A	B	A	A
RIL34	13.45	B	H	H	B

Lg	A2	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	SCN
cM		4.2	4.3	4.3	4.3	4.3	4.3	4.5	4.6	4.7	6.7	7.7	8.6	10.9	12.7	52.9	
NIL#	BARC-BLT65	SIUC-Sat_001	SIUC-Sac13	SIUC-TMD1	SST-10893	SIUC-Satt24	SIUC-Satt37	BARC-Satt309	SIUC-Satt27	SIUC-ATG4	SIUC-OIO3	SIUC-CTA13	SIUC-Satt122	BARC-Satt610	BARC-Satt570	BARC-Satt594	Female index
B. NILs																	
22	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	1.68
38	B	B	B	B	B	B	B	B	A	A	H	B	B	B	A	B	2.00
23	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**B**	**2.02**
30	B	B	B	B	B	B	B	B	B	A	B	B	B	B	A	B	2.02
10	B	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	2.10
7	B	B	H	H	H	H	H	A	A	A	A	A	A	A	A	B	2.18
2	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	2.44
18	B	B	H	H	H	H	H	B	B	B	B	B	B	B	B	B	2.52
5	B	B	B	B	B	B	B	B	B	B	H	B	H	B	B	B	2.61
24	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	3.36
35	B	B	B	B	B	B	B	B	B	A	B	B	B	B	A	B	3.45

Table 2 continued

Lg	A2	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	SCN
cM		4.2	4.3	4.3	4.3	4.3	4.3	4.5	4.6	4.7	6.7	7.7	8.6	10.9	12.7	52.9	
NIL#	BARC-BLT65	SIUC-Sat_001	SIUC-Sac13	SIUC-TMD1	SST-10893	SIUC-Satt24	SIUC-Satt37	BARC-Satt309	SIUC-Satt27	SIUC-ATG4	SIUC-OIO3	SIUC-CTA13	SIUC-Satt122	BARC-Satt610	BARC-Satt570	BARC-Satt594	Female index
21	B	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	3.45
1	B	B	H	H	H	H	H	H	H	B	B	B	B	B	B	B	5.99
40	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	6.22
19	B	H	H	H	H	H	H	H	H	B	B	B	H	H	A	B	28.66
6	B	B	H	H	H	H	H	B	B	B	B	B	B	B	B	B	29.24
4	B	B	H	H	H	H	H	B	B	B	B	B	A	H	A	B	31.76
33	**B**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**H**	**A**	**B**	**39.71**
13	B	A	A	A	A	A	A	H	A	A	H	B	A	A	A	B	56.83
26	B	A	A	A	A	A	A	H	H	A	B	B	B	B	A	B	72.86
3	**B**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**B**	**80.59**

Resistance was determined with the PA3 isolate of HgType 0. The resistance to SCN was associated with segregation at Sac13, TMD1 and the three SNP markers. The resistance allele of the *Rhg1* locus was co-dominant with the PA3 HgType 0. Female index was the mean of five plants repeated once (10 plants total). Three contrasting genotypes were highlighted in *bold*

A the Essex allele; *B* the Forrest allele and *H* lines that contained both heterogenous and heterozygous plants (e.g. NIL34-33)

variations (HSVs; Saini et al. 2008). In the NIL population the RLK alleles were distinguished using two SNPs from the LRR region (506 and 1040; Fig. 3); and the SIUC-Satt-TMD1 marker (Fig. 4; Afzal and Lightfoot 2007). In the RLK to laccase intergeneic region STS probe 10893 was used (Fig. 1). In the laccase to antiporter intragenic region was SUC-Satt 24. In the first antiporter to second antiporter intragenic region probe SIUC-Satt37 was used. Each probe could detect polymorphism among the alleles of the three genes at *rhg1* in the ExF derived NILs but not at the paralogous loci.

Evidence for the absence of recombination events at *rhg1*

Using this set of probes no recombination events have been found between TMD1 and SIUC Satt75 in the resistant haplotype within the region encompassing the three genes at *rhg1* (Fig. 1; Table 1). Negative results were notoriously difficult to delimit. However, as well as the lines reported here, used have been the E × F RIL population ($n = 100$; Meksem et al. 2001a, b, c; Lightfoot et al. 2005); NILs collected from two populations ExF 34 ($n = 2{,}000$) and E × F 11 ($n = 2000$; Triwitayakorn et al. 2005); RILs collected from the R × H and F × H populations ($n = 975$; Prabhu et al. 1999); the set of SCN resistant PIs ($n = 112$; Ruben et al. 2006); and individual NIL plants of the heterozygous line E × F 34-33 ($n = 200$). Here, the region from the RLK to the Na H antiporter was analyzed with three markers (Hyten et al. 2007) polymorphic in E × F (Fig. 1;Table 1). Again no recombination events were found, even though not all lines expressed a functional resistance allele at the *rhg1* locus on LG G. The region is near a telomere and the genes within it are all expressed. Therefore, it is unlikely to be a heterochromatic region suppressing recombination. Instead, the kinase domain of the RLK at *Rhg1* was inferred to be a gamete or zygote killer element and the hypothetical protein was proposed as the target locus kept in the resistant state when the RLK allele conferred SCN resistance (Fig. 2). The laccase trapped between these two gene and the intergenic regions are held in phase by the locus interaction.

The zygote or embryo lethal gene on Lg M that is co-inherited with *rhg1* (Webb et al. 1995) mapped near Satt594 in ExF (Table 2; Fig. 2). Satt594 usually maps to Lg G but HSVs exist on Lg M. The locus was fixed to the R haplotype in the NILs. Here we propose the locus be named suppressor of *rhg1* or *sup-rhg1*.

Dominant, recessive or co-dominant nature of *rhg1*

Both recessive and co-dominant roles have been assigned for the *rhg1* locus. In both past and recent studies with PIs, resistance encoded by *rhg1* was reported as recessive (Arelli et al. 2000; Brucker et al. 2005) whereas previously in NILs, the *rhg1* locus was reported to be co-dominant (Meksem et al. 2001a) but without single-plant marker allele associations. Clearly, co-dominant and recessive roles of plant disease resistance loci are rare and unusual (Li et al. 2006; Nieto et al. 2007). On the basis of the segregation pattern at the intrageneic TMD1 (intron) and Sca5 (promoter) markers, in NIL 34-33 background, the *rhg1* locus was shown to be dominant (Fig. 3; Table 2).

Table 3 Cyst score TMD1 genotype for plants from Fig. 2 using the JB3 isolate of Hg Type 0

Lane	Score	No of cysts	Phenotype
1	A	220	Susceptible
2	A	210	Susceptible
3	A	237	Susceptible
4	A	152	Susceptible
5	A	167	Susceptible
6	B	012	Resistant
7	A	198	Susceptible
8	B	017	Resistant
9	B	017	Resistant
10	A	247	Susceptible
11	A	183	Susceptible
12	A/B	012	Resistant/heterozygous
13	A	224	Susceptible
14	A	177	Susceptible
15	A/B	026	Resistant/heterozygous
16	B	000	Resistant
17	A	209	Susceptible
19	A	187	Resistant[a]
18	B	014	Susceptible[a]
20	A/B	020	Resistant/heterozygous
21	B	021	Resistant
22	A	176	Susceptible
23	A	186	Susceptible
24	A	171	Susceptible
25	A	144	Susceptible
26	B	018	Resistant
27	A	184	Susceptible
28	A	206	Susceptible
29	A/B	023	Resistant/heterozygous
30	A	218	Susceptible
31	B	043	Resistant
32	A	172	Susceptible
33	A	233	Susceptible
34	A	168	Susceptible

The *Rhg1* locus was dominant

A the Essex allele; *B* the Forrest allele and *H* heterozygous plants

[a] Plants were switched in order

In NIL 34-33 segregating at the *rhg1* locus, four plants were heterozygous at the TMD1 (Fig. 4; Table 2) and Sac5 markers (results not shown). The cyst scores, for each plant in the NIL corresponded with the respective alleles at the *rhg1* locus. For plants polymorphic at TMD1, the cyst score correspond to those for resistant plants. Therefore, the *rhg1* locus was dominant in this set of NILs with this SCN population.

The discrepancies in dominance among different populations may be associated with the genetic background the gene resides in and may result from interactions among genes at the *rhg1* locus and/or modifier genes at other loci (Webb et al. 1995; Fig. 2). The phenotype at the rice blast resistant locus, *xa3* is also influenced by the genetic background. The gene at the locus behaves differently in different genetic backgrounds, even displaying dominance reversal in one case (Xiang et al. 2006).

Inhibition of root growth by alleles of rhg1 in the NILs

When counting the cysts with prior knowledge of the allele it was noted that root mass and vigor appeared to differ among genotypes. Measurements of root mass showed a significant difference among NILs that was associated with the allele at *rhg1* or linked loci (Fig. 4; Table 4). Across several experiments, both NILs that were pure breeding susceptible and NILs that segregated some susceptible lines had higher root masses than their SCN resistant counterparts. This phenomenon might underlie the global association of resitance to SCN with low seed germination, seedling vigor, stand formation and ultimately seed yield.

The recombination events found among the six Hg type 0 susceptble PIs (Ruben et al. 2006) suggested that the action of *rhg1* requires elements to the distal side of the RLK intron, possibly one or all of the 3 polymorphisms found in the intracellular kinase of the complete RLK. Some mutations in the kinases of other plant RLKs are known to be lethal (Lease et al. 2001; Wang et al. 2005). Kinase mutants can be lethal in many cases (Sessa et al. 2000; Kollewe et al. 2004). Therefore, it may be the kinase at the *rhg1* locus that underlies restricted root growth in resistant genotypes directly or after some sort of interaction.

Genes at *rhg1* were expressed in both resistant and susceptible soybean roots

The presence of the RLK at *rhg1* mRNA and protein in roots was confirmed by RT-PCR and Western hybridization (Fig. 5). The *rhg1* transcript was detected under both inoculated and non-inoculated conditions in the both the resistant cultivar Forrest and resistant NIL34-23 and the susceptible cultivar Essex and susceptible NIL34-3. The quantitative PCR used to determine differences in transcript abundance between infected and uninfected cultivars showed the mRNA was increased about twofold following SCN inoculation.

Expression judged by examination of EST libraries in silico, cDNA libraries by hybridization and mRNA populations by RT-PCR showed the RLK (Fig. 5) and laccase (Iqbal et al. 2008) were transcribed in both non-infested roots and SCN-infested roots. However, paralogs were detected for each gene as judged by multiple amplicons from cDNA with laccase probes (G10; Iqbal et al. 2008) and antiporter probes (G11; not shown). Therefore, genes

Table 4 Association of mean root growth in NILs with resistance to SCN and SDS in seedlings at 28 days after germination with SCN infestations or *F. virguliforme* infestations

NIL	Root					SCN	SDS
	Allele	Mass (g)	Significant differences	Range (g)	*n*	FI (%)	DS
34-3	SI	1.99 ± 0.21	ab	1.48–2.44	11	102 ± 14	3.0 ± 0.9
34-23	RI	0.98 ± 0.14	c	0.43–1.61	13	7 ± 3	1.0 ± 0.1
34-33-1	RI	1.64 ± 0.18	bc	0.97–2.24	11	8 ± 3	1.1 ± 0.2
34-33-2	SI	2.38 ± 0.28	ab	1.66–3.6	11	84 ± 17	3.3 ± 0.8
34-33-3	HI	1.69 ± 0.19	bc	1.48–1.98	4	9 ± 5	2.2 ± 0.4

Female index (FI) was a percentage of cysts of Hg Type 0 found compared to a susceptible line. Disease severity (DS) was a 1–9 scale for the leaf scorch caused by *F. virguliforme* characteristic of sudden death syndrome (SDS)

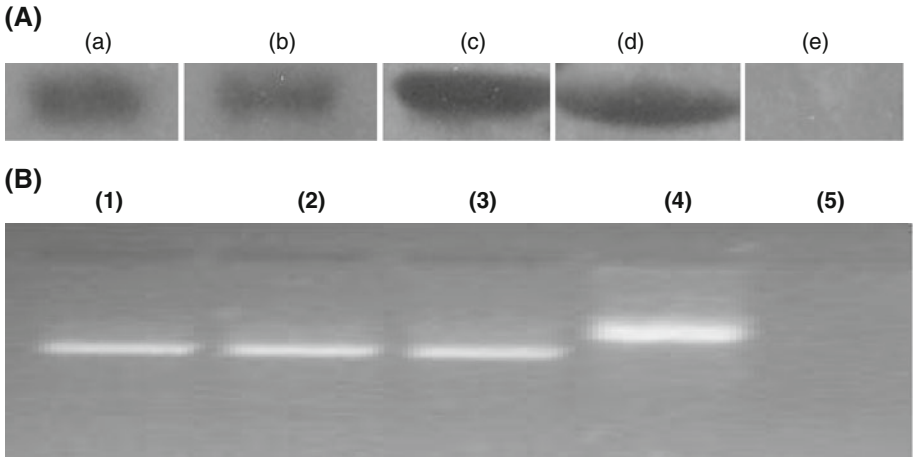

Fig. 5 Expression of the RLK at *rhg1* in Soybean Roots. **A** Western hybridization using an anti-RHG1 antibody from; Forrest root (*a*); Essex root (*b*); expressed RHG1-LRR-Shrt (*c*); expressed RHG1-LRR-Long (*d*); and expressed RHG 4 (*e*). **B** Agarose gel electrophoresis of cDNA amplified using *rhg1* LRR flanking primers from RIL 34-23; non-infested control (*1*); SCN-infested RIL 34-23 (*2*); SCN-infested RIL 34-3 (*3*); and Forrest genomic DNA (*4*). Negative control without template is shown in *lane 5*

in the *rhg1*-like paralog(s) might influence *rhg1* activity by cooperation or competition.

Discussion

The paralogs raised significant barriers to reverse genetic approaches to provide unequivocal proof that the RLK at *rhg1* candidate gene underlies part of the resistance to Hg type 0 (Melito et al. 2010). A second barrier were the genes that interact with *rhg1* including *sup-rhg1*, and *Rhg4* in this material (Fig. 2). A third barrier was the role of *rhg1* in normal plant development that can be inferred from the restricted root growth of NILs (Fig. 4; Table 4), some mutants in this gene (K. Meksem, unpublished data) and the shoot effects measured in grafting experiments (Afzal et al. 2009). Proof of RHG1 function may require knock-outs of each of the three paralogs. Stable transformation of the RLK to a new location has been shown to provide partial resistance to SCN and full resistance to SDS (Srour et al. 2011; Lightfoot and Meksem 2010). However, because the resistance to SCN was only partial roles for the laccase, antiporter and other genes at the *Rhg1/Rfs2* locus cannot be discounted.

The probes developed provide a high throughput alternative to satellite markers for marker assisted selection, three allelic discrimination tools were developed for Taqman primer probes. The first Taqman probe (1040) could successfully discriminate resistant types 1 and 2 from susceptible haplotypes 2, 3 and 4. Marker 506 could distinguish R types 2 and 3 from other haplotypes. Marker 2050 distinguished among susceptible types.

The data presented here suggests the genes linked to the primary candidate the RLK may encode factors involved in the modulation of *rhg1* activity. Possible roles include contributions to additive resistance; contributions to resistance in other resistance types (e.g PI88788 and Toyohazu; R types 2 and 3) or contributions to the resistance to other Hg types (Nelsen et al. 2003; Li et al. 2009). Unlikely, in view of the susceptibility of segregation events within the interval from the RLK to Satt309 in both PI evolution and NIL segregation, is the hypothesis that the linked genes are factors necessary in susceptible genotypes for SCN parasitism. The dominance of *rhg1* in NIL segregation also suggests the genes are active in resistant types and inactive in susceptible genotypes. In conclusion, the *rhg1* locus was inferred to be a complex of from three to several genes

assembled and co-inherited over long periods of selection for resistance to a pandemic pest, root parasitic nematodes.

Acknowledgments The physical map was based upon work supported by the National Science Foundation under Grant No. 9872635. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. The continued support of SIUC, College of Agriculture and Office of the Vice Chancellor for Research to DAL is appreciated. The authors thank Dr. Q. Tao and Dr, H.B. Zhang for assistance with fingerprinting. We thank Dr. C. Town and Dr. C. Foo at TIGR for the BES. We thank the DOE CJSP for release of the WGS reads and scaffolds. We thank the Government of India for support of the Fellowship for NS.

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