

Resistance to Soybean Cyst Nematode: *Rhg1*

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Abstract

The genes underlying *rhg1* lie at a sometimes dominant sometimes co-dominant locus, necessary for resistance to all Hg types of the soybean (*Glycine max* (L.) Merr.) cyst nematode (*Heterodera glycines*). Genomic research identified; nucleotide changes within a candidate gene encoding a receptor like kinase (RLK) that were capable of altering root development and thereby part of the resistance to Hg types 0 (race 3); changes in a laccase that are capable of altering cyst development; and genes underlying changes in membrane biology. This set of three genes are subject to co-selection with a modifier locus on another linkage block. Root development is slowed in the resistant seedling and results in end of season yield loss when SCN is not present. However, in the presence of SCN resistant seedling roots grow just as vigorously as the now slower growing parasitized susceptible roots and therefore show little loss to SCN parasitism. In some genotypes but not others the RLK can act alone to confer resistance. Functional paralogs of the three gene cluster have been found on other linkage groups including A1, B1, G, and O and these can be functional in different sources of resistance like *G. soja*, PI 437654 and PI438489B. At *rhg1* the allele differences change the structure, interacting partners and activity of the LRR protein and the laccase. The changes between the alleles result in about 30 other proteins (judged by 2 D gels), 112 metabolites (by FTICRMS) and 8 metabolites (by

GCMS) to increase in abundance in roots during SCN infection in the resistant NILs. Understanding the basis of root stunting by resistance alleles will be used to improve methods for developing new nematode resistant soybean cultivars that do not suffer from the yield suppression and low seed germination rates of existing cultivars.

Keywords: Soybean cyst nematode (SCN), nematode resistance, proteomics, soybean roots.

Introduction

Soybean [*Glycine max* (L.) Merr.] is the world's most important legume crop, it is grown for both oil and protein co-products. Many natural by-products are also derived from soybean and several possess significant antimicrobial and pharmacological properties (Dixon and Sumner, 2003). The abundance and variety of soybean's bioactive factors suggest the species has evolved and been domesticated in the presence of many persistent pathogen populations.

After water deficit, the leading causes of seed yield losses in soybean are biotic stresses from pathogen infections (Wrather et al., 2001). Amongst soybean pathogens, the soybean cyst nematode (SCN) causes the most damage. Yield losses in the

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world due to SCN infection have approached \$2 billion a year (Niblack et al., 2004) and in the US alone amounted to 1.5 billion for the 1996-1997 period (Chen et al., 2001). SCN preventative measures include crop rotation, the use of resistant cultivars and nematicides. The use of resistant cultivars provides the most efficient and practical control of SCN.

Soybean breeding has used heavily three plant introductions (PIs) resistant to the cyst nematode (Brucker et al., 2005b). The PIs 'PI88788' 'Peking' and 'PI437654' have each been shown to carry resistance loci effective against multiple nematode races. However, the resistance to SCN is quantitative and controlled by multiple loci. Previously identified were two quantitative trait loci that provided substantial resistance against the soybean cyst nematode Hg Type 0 (a biotype of race 3) in NILs derived from Peking via 'Forrest' (Meksem et al., 2001; Ruben et al., 2006). The resistance locus *Rhg4* on linkage group A2 provided resistance against Hg Type 0 whereas *rhg1*, the resistance locus on linkage group G provided some resistance against nematodes of all known races. The *rhg1* locus allele from Peking causes a rapid necrosis of the giant cells and the formation of thickened cell walls around the defunct feeding site (syncytium) by 24-48h after the SCN female starts to feed, some 4-5 days after infestation of the roots (Alkharouf et al., 2004, 2006; Ithal et al., 2007a,b). The activity of the resistance allele is associated with global changes in transcript abundance (TA) within the syncytium and systemic changes in the root system with hundreds of transcripts altered. However, in plants the correlations between transcript, protein and metabolite abundances are low. Therefore, key proteins may have been overlooked by transcript analysis. Further, some proteins can alter the metabolome without alterations in their abundances (Edwards, 1996; Ito et al., 2003; Schad et al., 2005; Mungur, 2008). Therefore, key metabolites may remain undetected. Finally, protein-protein and protein-metabolite interactions are important in many plant responses to the environment (Geisler-Lee et al., 2008). Therefore, key interactions may remain undetected by the transcript analyses reported to date.

The *rhg1* locus appears to be a multifunctional and multigenic locus (Ruben et al., 2006; Iqbal et al., 2009; Afzal et al., 2009a). The locus contained several genes capable of altering the transcriptome, proteome, metabolome and interactome including; a receptor like kinase inferred to alter root development (RLK; Afzal and Lightfoot, 2007; Afzal et al., 2008a,b; 2009a); an unusual laccase involved in phenolic metabolism (Iqbal et al., 2009); and a predicted Na/H antiporter. Flanking the locus were two proteins of unknown function, one of which is transcribed in roots (Triwitayakorn et al., 2005; Ruben et al., 2006). Each of the genes was encoded different alleles with significant amino acid and nucleotide changes in resistant soybean compared to susceptible soybean (Ruben et al., 2006; Afzal et al., 2008b; 2009a). Analysis in near isogenic lines suggests the resistance alleles of the core set of three linked genes cannot be separated by recombination without the loss of resistance (Afzal, 2007; Afzal et al., 2008b; 2009a). Key elements of that hypothesis are reviewed in this paper.

Materials and Methods

Plant Materials

The genetic materials and methods used were described in Triwitayakorn et al. (2005), Ruben et al. (2006), Afzal et al. (2008; 2009a,b, 2012). Briefly, the seeds of RILs and the NIL populations derived from the cross of Essex by Forrest 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. Seeds of NIL 34-23 (resistant haplotype between markers Satt 214 to Satt 570) NIL34-33 (heterogeneous and heterozygous across the interval) and NIL 34-3 (susceptible haplotype across the interval) were used at the F5:13 generation.

Seedlings were grown in a growth chamber and growth was analyzed as described by Afzal et al. (2012). DNA extractions and marker analysis also followed the methods of Afzal et al. (2012).

Results

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., 1990; Brucker et al., 2005b) 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 intragenic TMD1 (intron) and Sca5 (promoter) markers, in NIL 34-33 background, the *rhg1* locus was shown to be dominant (Figure 1; Table 1).

In NIL 34-33 segregating at the *rhg1* locus, 4 plants were heterozygous at the TMD1 and Sac5 markers. The cyst scores, for all plants in the NIL population 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. Genotypes were *rhg1-e/rhg1-e Rhg4-p/Rhg4-p* for NIL 34-3 and *Rhg1-p/Rhg1-p Rhg4-p/Rhg4-p* for NIL 34-23 whereas NIL34-33 contained both those and *Rhg1-p/rhg1-e Rhg4-p/Rhg4-p* in different plants. 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).

Table 1. Cosegregation of alleles of the marker TMD1 in the RLK at *rhg1* and co-dominant resistance to SCN.

Plant/Line	Score	Cyst No.	Phenotype
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
18	B	014	Resistant*
19	A	187	Susceptible*
20	A/B	020	Resistant/Heterozygous
21	B	021	Resistant
22	A	176	Susceptible

The recombination events found among the six Hg Type 0 susceptible PIs (Ruben et al., 2006) suggests 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., 2006). 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.

Syntenic Paralog of the *rhg1* Locus

To identify *rhg1* paralogs, primers specific to the conserved regions of *rhg1* leucine-rich repeat and *rhg1*-kinase domain were designed and the genome sequence examined. 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 antiport-

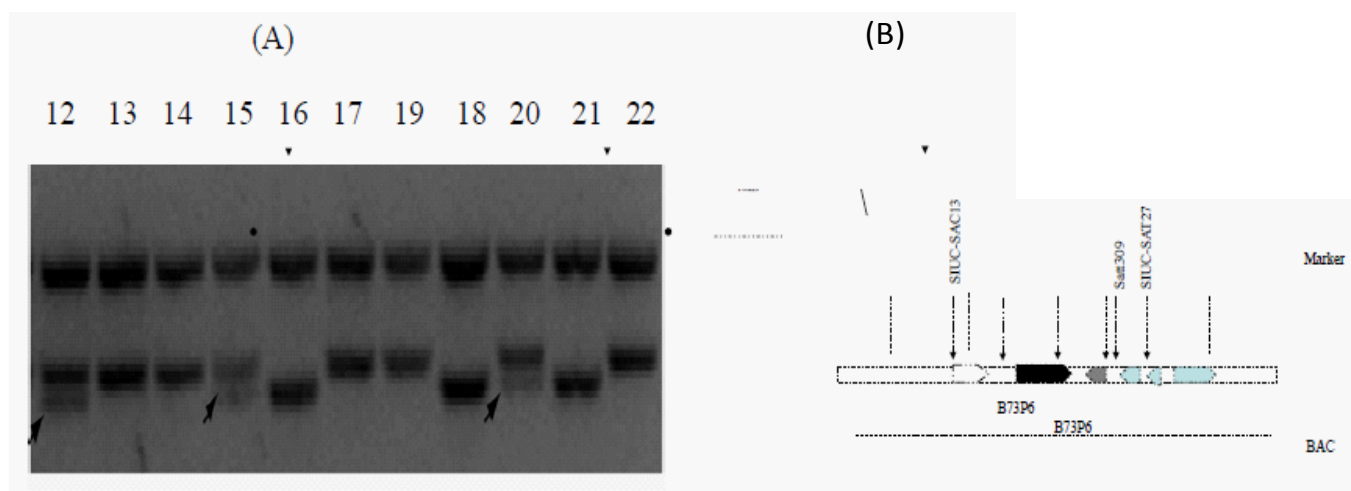


Figure 1. Analysis of NIL 34-33 with TMD1 and other markers. Panel A; Out of the 34 plants analyzed in this run (10 shown), 4 were heterozygous (Lanes 12, 14, 20 and 29). The four heterozygous plants had a resistant phenotype (arrowed). The upper invariant band is from the RLK paralog on LGO, the lower band is from the intron of the RLK on LG G. SCN counts and gel scores for the plants are given in Table 1. Plants 18 and 19 were mislabeled. Panel B Marker map of the genomic region around *rhg1* showing TMD1 position and locus ideogram showing the locations of genes.

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 (Figure 2; Table 3). 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 resistance to SCN with low seed germination, seedling vigor, stand formation and ultimately seed yield.

ers, the kinase and the helicase; Figure 3). The DNA markers sequence paralogs were present but more diverged. Sequence analysis of the alleles in Williams 82 and Forrest RLK at *rhg1* showed 99% amino sequence identity. Among the paralogs DNA sequence identity was high (~92% in geneic region; Supplemental Figure 1). Amino acid identity was 84% in the LRR, 86% in the transmembrane domain and 94% in the kinase domain. The laccase and the antiported also shoed 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., 1997) and LG B1 (the syntenic cluster ; Wu et al., 2009).

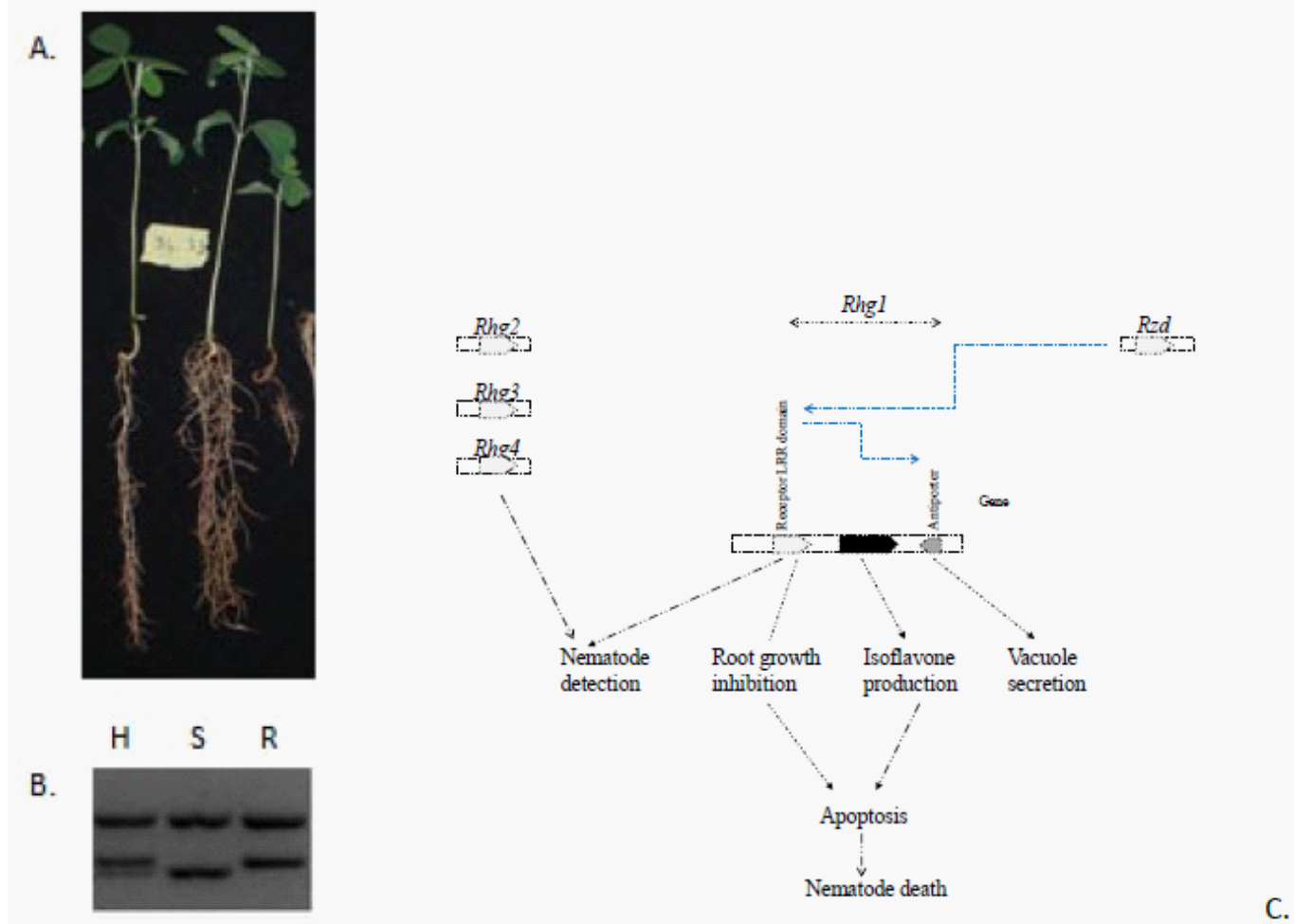


Figure 2. The RHG1 protein alters root development. Panel A; Soybean NILs at 2 weeks after planting but pre SCN inoculation show different root morphologies, post inoculation root masses are not different (by 6 weeks). Therefore, *rhg1* inhibits germination and early root growth. Panel B; the root morphologies cosegregated with the allele in the RLK at the *rhg1* locus as shown by the intragenic marker TMD1 (satellite in the intron). Panel C; 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 3 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 suppression of the RLK.

The paralogs may encode proteins that recognize novel race biotypes or substitute for *rhg1* following activation in certain PIs or crosses.

Examining the *rhg1* Interactome

Two dimensional gel electrophoresis that resolved more than 1,000 protein spots on each gel showed *rhg1* controlled the abundance of only thirty (Afzal et al., 2009b). The proteins were grouped into six functional categories. Metabolite analysis by GC-MS identified 131 metabolites among which 58 were altered by one or more treatment, 28 were involved in primary metabolisms. Taken together the data showed seventeen pathways that were altered by *rhg1* controlled metabolisms associated with SAR-like responses including xenobiotic, phytoalexin, ascorbate and inositol metabolism as well as primary metabo-

lisms like amino acid metabolism and glycolysis. The pathways impacted by the *rhg1* allelic state and SCN infestation agreed with transcript abundance analyses but identified a smaller set of key proteins.

Six of the proteins lay within the same region of the interactome identifying a key set of 159 proteins inferred to be involved in nuclear protein transport and degradation. The predicted interactome showed the chaperonin, F1-ATPase, multi catalytic endopeptidase, thaumatin-like protein, cytosolic heat shock protein and triose phosphate isomerase were interacting indirectly through 2 to 7 intermediates (Figure 4). The cluster of all the potential interacting neighbors showed 159 proteins in total, with 194 interactions. The protein functions included transcription factors, chaperonins, signal transduction factors and metabolisms involved in energy generation (Table 1). The four proteins in the cluster with most interacting partners were a GTP

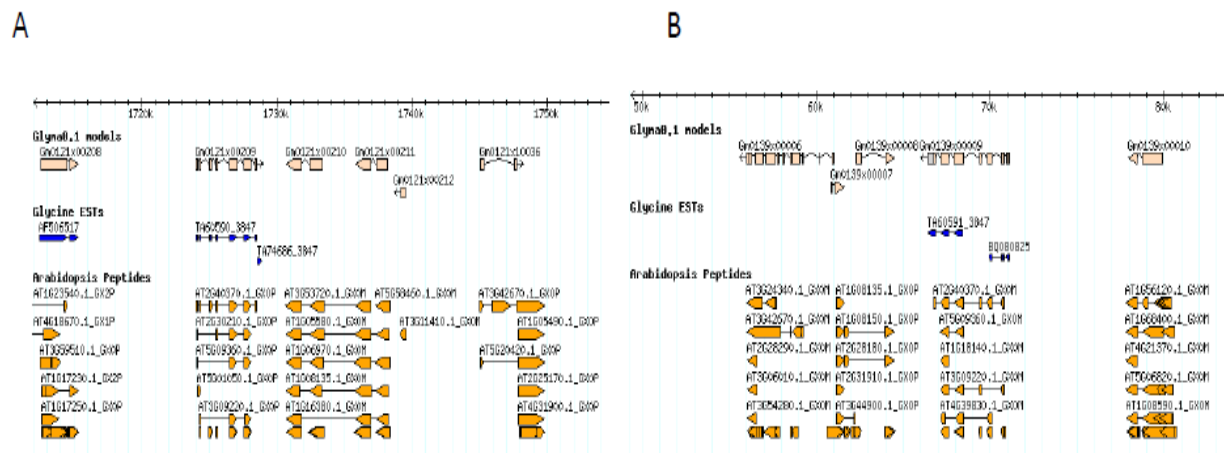


Figure 3. GBrowse view of 40 Mbp encompassing the *rhg1* locus from Lg G and the syntenic gene cluster from LgB1 in Williams 82. The image was viewed at Phytosome.net in 2008. Panel A shows the region around *rhg1* on scaffold 121 (LgG). Panel B shows the region around the *rhg1* paralog on scaffold 139 (Lg B1). Note gene orders are the same but the scaffold orientation is inverted. Panel C shows the alignment of the two DNA sequences and Panel D the amino acid alignments centered on the RLK at *rhg1*.

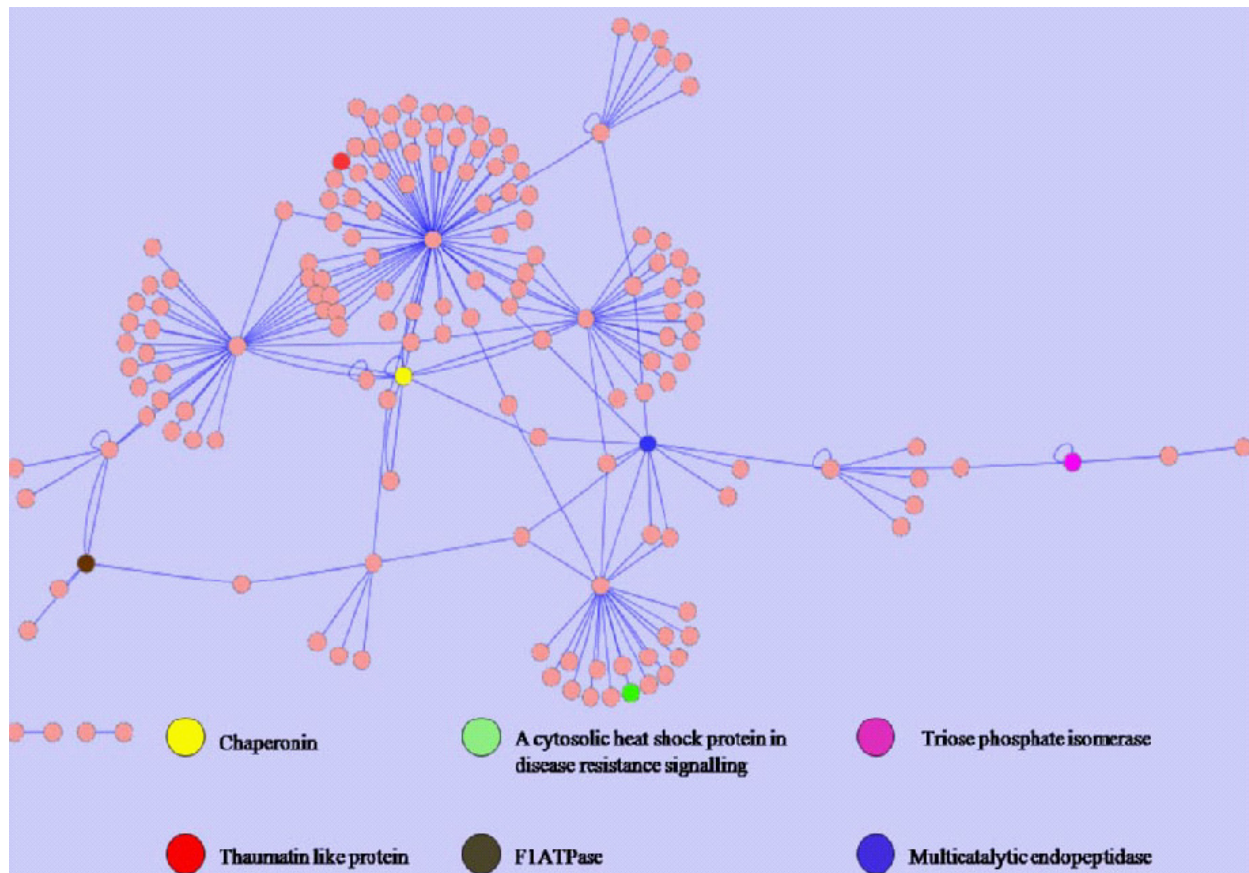


Figure 4. A part of the predicted interactome for soybean showing six proteins from the 2D gel analysis (chaperonin, F1ATPase, multi catalytic endopeptidase, thaumatin like protein, cytosolic heat shock protein and triose phosphate isomerase). The interactome was extended by one node to include 159 nodes (proteins) and 194 edges (interactions). Two proteins with a previously characterized role in disease resistance were identified within this part of the interactome; the thaumatin like protein induced in response to fungal pathogens (blue) and a cytosolic heat shock protein in disease resistance signaling (green). All six proteins interacted through 2-7 intermediate partners (see Supplemental Table 1 Afzal et al. (2009b) for pink spot identification numbers). Auto-regulation was predicted for the triose phosphate isomerase and the chaperonin.

binding-family protein (28 partners), importin alpha-2 subunit (64 partners), importin beta-2 subunit (22 partners) and a 26S proteasome non-ATPase regulatory subunit 3a (21 partners). Therefore, the regulation of protein degradation and transport of proteins across the nuclear membranes may be important aspects of resistance to SCN.

Finally, two proteins (glucose 6 phosphate isomerase, EC 5.3.1.9; and isoflavone reductase, EC 1.3.1.45) and two metabolites (maltose and unidentified) differed in resistant and susceptible NILs without SCN infestation and may form the basis of a new assay for the selection of resistance to SCN in soybean.

Conclusions

The data presented here suggests the genes linked to the primary candidate 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 (eg PI88788 and Toyohazu; R types 2 and 3) or contributions to the resistance to other Hg types (Nelsen et al., 2003). 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 coinherited over long periods of selection for resistance to a pandemic pest, root parasitic nematodes. The genes in the loci were shown to regulate pathways involved in protein trafficking, protein degradation and primary metabolisms. Transgenic plants will be used to tease apart the layered functions in the next phase of this research.

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