Detection of rare nematode resistance Rhg1 haplotypes in *Glycine soja* and a novel Rhg1 α-SNAP

Derrick J. Grunwald | Ryan W. Zapotocny | Seda Ozer | Brian W. Diers | Andrew F. Bent

Abstract

This study pursued the hypothesis that wild plant germplasm accessions carrying alleles of interest can be identified using available single nucleotide polymorphism (SNP) genotypes for particular alleles of other (unlinked) genes that contribute to the trait of interest. The soybean cyst nematode (SCN, *Heterodera glycines* [HG]) resistance locus *Rhg1* is widely used in farmed soybean (*Glycine max* (L.) Merr.). The two known resistance-conferring haplotypes, *rhg1-a* and *rhg1-b*, typically contain three or seven to 10 tandemly duplicated *Rhg1* segments, respectively. Each *Rhg1* repeat carries four genes, including *Glyma.18G022500*, which encodes unusual isoforms of the vesicle-trafficking chaperone α-SNAP. Using SoySNP50K data for *NSF* allele presence, we discovered a new *Rhg1* haplotype, *rhg1-ds*, in six accessions of wild soybean, *Glycine soja* Siebold & Zucc. (0.5% of the ∼1,100 *G. soja* accessions in the USDA collection). The α-SNAP encoded by *rhg1-ds* is unique at an important site of amino acid variation and shares with the *rhg1-a* and *rhg1-b* α-SNAP proteins the traits of cytotoxicity and altered N-ethylmaleimide sensitive factor (NSF) protein interaction. Copy number assays indicate three repeats of *rhg1-ds*. *G. soja* PI 507613 and PI 507623 exhibit resistance to HG type 2.5.7 SCN populations, in part because of contributions from other loci. In a segregating *F₂* population, *rhg1-b* and *rhg1-ds* made statistically indistinguishable contributions to resistance to a partially virulent HG type 2.5.7 SCN population. Hence, the unusual multigene copy number variation *Rhg1* haplotype was present but rare in ancestral *G. soja* and was present in accessions that offer multiple traits for SCN resistance breeding. The accessions were initially identified for study based on an unlinked SNP.

1 INTRODUCTION

Productive enhancements in feed and food crops are often driven by phenotypic selection during conventional plant breeding, but phenotype alone does not always reveal the donor material with the best potential for trait diversification.
and improvement (Tuberosa, 2012). Recent developments, such as single nucleotide polymorphism (SNP) genotyping and next generation sequencing have made it possible to pre-screen germplasm for desirable traits through correlation with sequence information (Poland et al., 2012). In some cases, this approach can be extended to wild relatives of crop plants. Novel alleles of loci of agronomic importance may then be subjected to functional characterization. Alleles of interest can be moved into elite varieties via sexual crosses or transgenic approaches.

Soybean [Glycine max (L.) Merr.] is one of the world’s most important legume crops, providing a major source of vegetable oil, protein meal for animal feed, and potential sources of renewable energy (http://soystats.com/) (Graham & Vance, 2003; Stacey, 2010). Soybean cyst nematode(SCN) (Heterodera glycines [HG]) is the most economically damaging pathogen of soybean, routinely causing upwards of US$1 billion each year in the United States alone (Davis et al., 2008; Koenning & Wrather, 2010; Mitchum, 2016). Soybean cyst nematode eggs can remain viable for years in cysts, which are recalcitrant to many environmental or chemical conditions, making control of established SCN populations difficult (Niblack et al., 2006). Resistant varieties and crop rotation are the major methods of SCN control. Glycine soja is the wild ancestor of cultivated soybean, and the more diverse gene pool in G. soja species vs. domestic soybean offers a source of novel alleles or genes for traits of agronomic interest including SCN resistance (Hyten et al., 2006; Liu et al., 2020).

The strong-effect quantitative trait locus (QTL) Rhg1 (Resistance to Heterodera glycines I) is the most used source of SCN resistance, with the rhg1-b haplotype from PI 88788 present in the vast majority of SCN-resistant soybean grown in the United States. (Colgrove & Niblack, 2008; Concibido et al., 2004; Donald et al., 2006. Intriguingly, Rhg1 is a tandemly repeated block of four genes, and the number of repeats varies from one in susceptible soybean varieties or three in most rhg1-a haplotypes to nine or 10 copies in rhg1-b haplotypes (Cook et al., 2012). None of the genes in the repeated block are reminiscent of canonical defense or disease resistance genes. Both silencing and overexpression experiments have established a role in SCN resistance for three of the four genes in the Rhg1 locus, one of which (Glyma.18G022500) encodes an α-SNAP (alpha-soluble N-ethylmaleimide sensitive factor (NSF) attachment protein) (Cook et al., 2012).

The α-SNAP is a functionally conserved eukaryotic protein that interacts in multimeric complexes with both NSF and soluble NSF attachment protein receptor (SNARE) proteins to mediate vesicular trafficking (Jahn & Scheller, 2006). In particular, α-SNAP and NSF cooperate to promote vesicle trafficking through their disassembly for recycling of the bundled v- and t-SNARE complexes that form during vesicle fusion (Jahn & Scheller, 2006). In soybean, we recently discovered that Rhg1-encoded α-SNAPs are unusual in that they bind poorly to wild-type NSF (Bayless et al., 2016). In Nicotiana benthamiana Domin transient assays, expression of rhg1-a or rhg1-b α-SNAPs disrupts vesicle trafficking and is cytotoxic, eventually causing cell death (Bayless et al., 2016, 2018). Furthermore, these aberrant α-SNAPs accumulate approximately tenfold in syncytial cells as a response to SCN, suggesting a role of vesicle trafficking efficiency in the outcome of SCN-soybean interactions (Bayless et al., 2016, 2019). Soybean haplotypes containing three Rhg1 repeats (low-copy [LC] haplotypes; rhg1-a; Peking-type or Hartwig-type) encode a distinct α-SNAP protein while those haplotypes containing nine to 10 Rhg1 repeats (high-copy [HC] haplotypes; rhg1-b; PI 88788-type) encode a second distinct α-SNAP protein (Cook et al., 2014). To maximize SCN resistance, varieties carrying LC rhg1-a haplotypes require presence of additional QTL at chromosome 11 (associated with a loss-of-function intron retention allele at a chromosome 11 α-SNAP gene) and at chromosome 8 (Rhg4, encoding a serine hydroxymethyltransferase) (Bayless et al., 2018; Lakhssassi et al., 2017; Liu et al., 2012). These and other studies (Lakhssassi et al., 2020; Liu et al., 2017; Patil et al., 2019; Yu et al., 2016) have identified additional attributes shared by or distinct between the rhg1-a vs. rhg1-b haplotypes.

Linkage disequilibrium has been observed between the soybean chromosome 18 Rhg1 locus and a chromosome 7 locus, with segregation distortion observed only in genotypes carrying an SCN resistance-associated Rhg1 allele (Webb et al., 1995; Kopisch-Obuch & Diers, 2005; Vuong et al., 2015). Our group recently found that this distortion is attributable to a unique NSF allele that is encoded at Glyma.07G195900 on chromosome 7 (Bayless et al., 2018). Termed NSF_RAN07 (for Rhg1-associated NSF on chromosome 7), this allele is present in 11% of 19,645 soybean accessions in the USDA collection but remarkably is present in all soybean accessions and segregating progeny that are homozygous for the SCN resistance-associated rhg1-a or rhg1-b haplotypes. At the apparent α-SNAP–NSF binding interface, the encoded NSF_RAN07
protein carries atypical amino acids at sites that become proximal to the unusual amino acids that distinguish α-SNAP<sub>Rhg1</sub>LC and α-SNAP<sub>Rhg1</sub>HC. The NSFRAN07 protein exhibits higher affinity than wild-type NSF for binding those resistance-associated α-SNAP proteins and enables the viability of soybeans carrying Rhg1-encoded SCN resistance (Bayless et al., 2018).

The gradual evolution of SCN populations toward HG types (or races) that partially or largely overcome the SCN resistance in commercially grown soybean varieties (McCarville et al., 2017; Niblack et al., 2008) has motivated searches for new SCN resistance sources. Specific <i>G. soja</i> accessions have already provided new sources of SCN resistance that show promise (Brzostowski et al., 2017; Usovsky et al., 2020; Yu & Diers, 2017). In a recent study by Lee et al. (2015b), whole-genome sequencing revealed but did not further investigate a tandem duplication of <i>Rhg1</i> in <i>G. soja</i> accession Jidong 5 (W06), suggesting that resistance-conferring <i>Rhg1</i> haplotypes may have arisen prior to the divergence of <i>G. max</i> and <i>G. soja</i>. Further research on <i>Rhg1</i> duplications in <i>G. soja</i> accessions can provide insights into the evolution of SCN resistance.

In the present study, we used a SNP marking the physically unlinked but genetically associated NSFRAN07 allele to discover and examine diversity of <i>Rhg1</i> in <i>G. soja</i> germplasm available through the USDA collection. We identified frequent <i>G. soja</i> accessions containing tandem repeat copies of the <i>Rhg1</i> locus that represent an apparent progenitor of soybean <i>rhg1-a</i> and <i>rhg1-b</i>. We found that the α-SNAP encoded at <i>G. soja</i> multicopy <i>Rhg1</i> loci is unique, yet carried structural and functional similarities to the <i>rhg1-a</i> and <i>rhg1-b</i> α-SNAPs. Partially as a result of contributions from other loci, specific <i>G. soja</i> accessions carrying NSFRAN07 and <i>rhg1-ds</i> were found to also carry strong resistance to problematic HG type 2.5.7 SCN.

## 2 MATERIALS AND METHODS

### 2.1 Plant materials

Seeds of 22 <i>G. soja</i> or <i>G. max</i> (the first 22 entries in Supplemental Table S1) were obtained from the USDA Soybean Germplasm Collection in Urbana, IL (https://www.ars-grin.gov/). LD10-10198 is an elite <i>G. max</i> experimental line developed at the University of Illinois that carries the <i>rhg1-b</i> haplotype for SCN resistance from PI 88788.

### 2.2 DNA extractions and oligonucleotide primers

Soybean genomic DNA was extracted from young cotyledons from respective <i>G. soja</i> or <i>G. max</i> accessions using standard CTAB extractions as in Keim et al. (1988) with modifications from Cook et al. (2012). The oligonucleotide primers used for polymerase chain reaction (PCR) and other assays are listed in Supplemental Table S3.

### 2.3 Copy number variation assays

Copy number variation assays were performed essentially as in Lee et al., 2015b. In brief, 80 ng of extracted genomic DNA was subjected to qPCR using primers directed against the bridge junction as in Figure 1 or <i>Glyma.18G022800</i> using SolisBio-dyne 5× Firepol (SolisBio-dyne; Cat. No. 08-36-00001). Copy number was estimated using the ΔCt method where ΔCt is the difference between <i>Glyma.18G022800</i> and bridge amplifications. For TaqMan assays, experiments were performed essentially as in Lee et al. (2016).

### 2.4 Targeted DNA extraction

DNA was enriched for the <i>Rhg1</i> locus using 837 specific patch oligos as in Varley et al. (2010) with significant modifications. DNA was first digested using three combinations of restriction enzymes: DraI/TaqI/NlaIII, MnlI/MboII, and Apol/HpyCH4V (100 ng DNA per reaction). A patch-driven ligation was performed with 2 nM of each complementary oligo (Supplemental Table S3), 40 nM nested forward, 20 nM ‘U2’, 20 nM ‘U2-Phospho’, 5 U of ampligase (Epicentre Cat. No. A3202K), and 1× ampligase buffer in 25 µl. This reaction was incubated for 10 min at 95 °C, followed by 30 sec at 95 °C and 4 min at 54 °C for 80 cycles. Undesired products and excess genomic DNA was then digested with the direct addition of 5 U exonuclease I (USB) and 100 U exonuclease III (Epicentre Cat. No. EX4425K). This was incubated at 37 °C for 1 h followed by inactivation at 95 °C. Products were purified using 42 µl (1.6x) of Agencourt AMPure XP beads (Beckman Coulter Cat. No. A63880) and eluted in 20 µl ddH2O. A nested PCR was then performed using KAPA HiFi HotStart Uracil+ ReadyMix (2x) (KapaBiosystems Cat. No. KK2801), 100 µM of the oligos ‘right-no homology’ and ‘left-no homology’, 2.5 mM MgCl<sub>2</sub> and 20 µl of the eluted product from previous step. This reaction was incubated according to manufacturer instructions with an annealing temperature of 66 °C and 30 s extension for 31 cycles. Product was again purified using 1.6× sample volume of Agencourt AMPure XP beads before submission for sequencing at the University of Wisconsin Biotechnology Center facility. Samples were individually barcoded before being sequenced using Illumina MiSEquation (Illumina) 2× 250 with 50% PhiX.
2.5 Sequence variant detection

Flow cell data were demultiplexed and Illumina adapter sequences trimmed by the University of Wisconsin Biotechnology Center facility. Universal sequences were trimmed using Cutadapt (v2.8; Martin, 2011). Previously published whole-genome sequencing read data from soybean HG type indicator lines as well as the *G. soja* genotypes W05 were also added to the analysis moving forward. (Cook et al., 2014, Bioproject PRJNA243933 [HG type test lines]; Xie et al., 2019, Bioproject PRJNA486704[W05]). Full genome alignment was performed as in Cook et al. (2014) using the Wm82.a2.v1 reference genome (Schmutz et al., 2010) using the programs BWA (v0.7.17, Li & Durbin, 2010) and Picard (v2.19). The HaplotypeCaller function with the GATK software package (Poplin et al., 2017) was used to detect variants in the genomic region Gm18:1632666-1663881. Because of the nature of our targeted DNA extraction, we lacked complete coverage of the locus. Because of this, we identified regions within *Rhgl* where all the sequenced *G. soja* samples had depth in excess of three. Vcftools (v1.13, Danecek et al., 2011) was used to pull out variants within these regions and filter for a depth of three and variant quality value of 50. A fasta sequence file was obtained for each sample using the
FastaAlternateReferenceMaker function within GATK. These fasta files were then imported to SplitsTree for network analysis (Huson & Bryant, 2006).

2.6 Phylogenetic analysis and phylogenetic trees

To determine the relationship between G. soja and G. max or among G. soja, we aligned all the SNPs available in the SoySNP50K database (Song et al., 2013, 2015). The program SplitsTree (v4.16.1) was used to perform the alignment and construct the network (Huson & Bryant, 2006). Uninformative sites were excluded in the network construction. Rooted phylogenetic trees were built using MEGAX (Kumar et al., 2018; Stretcher et al., 2020). The rooted phylogeny for the Rhg1 locus was performed using DNA sequences described in the preceding section, corresponding to bp Gm18:1,636,000-1,659,000 of G. max genome assembly version Glyma.Wm82.a2.

2.7 RNA isolation and cDNA synthesis

Total RNA was extracted from expanding soybean trifoliate leaves using either the DirectZol RNA miniprep plus kit (Zymo Research) or the RNeasy mini kit (Qiagen) using manufacturer’s instructions. RNA samples were DNase treated, and total RNA was quantified using spectrophotometry. Integrity of RNA was checked by visualization of rRNA bands on a 1.2% agarose gel. cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad) with ~1 μg RNA as input. The Peking soybean accession used in these studies was PI 548402.

2.8 Vector construction

The G. soja α-SNAP alleles (Glyma.18G022500) from each of the six multicycopy Rhg1 G. soja, and the NSF (Glyma.07G195900), SHMT Rhg4 (Rhg4; Glyma.08G11490), α-SNAP Ch11 (Glyma.11G234500), and 5’ NSF open reading frame (ORF) from G. soja accession PI 507623, were amplified from generated cDNA (for α-SNAP Rhg1, NSF and Rhg4), or genomic DNA (for α-SNAP Ch11 and 5’ NSF), using Kapa HiFi polymerase (Roche). They were placed directly under the control of the soybean ubiquitin promoter and nopaline synthase terminator in pBlueScript using Gibson assembly (Gibson et al., 2009) and subsequently sequence verified. For the α-SNAP Rhg1 and the NSF, the promoter-ORF-terminator expression cassette was digested with XbaI and PstI or NotI and Sall (New England Biosciences), respectively, and the fragments were purified using the Zymoclean large fragment DNA recovery kit (Zymo Research). Purified DNA fragments were then ligated into the pSM101 binary vector (Melito et al., 2010) using T4 DNA ligase (New England Biosciences).

For sequencing of the bridge junction, the NSF Ch07 amplicon and the last exon of α-SNAP Ch11 from G. soja Rhg1 bridge junction, NSF Ch07, or α-SNAP Ch11 amplicon was amplified from genomic DNA using GoTaq green (Promega) and cloned into pGEM-T Easy using ligation per manufacturers recommendations (Promega). Constructs were verified by colony PCR and diagnostic digest before being sequenced by Sanger sequencing.

2.9 Transient Agrobacterium expression in Nicotiana benthamiana

Agrobacterium tumefaciens strain GV3101 (pMP90) containing the indicated expression cassettes was infiltrated using a blunt tip tuberculin syringe at an OD600 = 0.8 into young leaves of N. benthamiana plants. The GV3101 cultures were grown overnight at ~28 °C in media containing 25 μg ml⁻¹ kanamycin and 100 μg ml⁻¹ rifampicin and induced for ~3 h in 10 mM MES (pH = 5.6), 10 mM MgCl2 and 100 mM acetosyringone prior to leaf infiltration. N. benthamiana plants were grown at 25 °C with a photoperiod of 16 h light at 100 μE m⁻² s⁻¹ and 8 h dark.

2.10 Antibodies and immunoblotting

Generation and validation of rabbit polyclonal antibodies raised against Rhg1 α-SNAP LC, Rhg1 α-SNAP HC, and α-SNAP WT was previously described in Bayless et al. (2016). Tissue preparation and immunoblots were performed essentially as in Bayless et al. (2016, 2018). Bradford assays were performed on each leaf or root extract supernatant and equal amount of OD395 total protein were loaded onto SDS–PAGE gels. Immunoblots for α-SNAP and NSF were incubated overnight at 4 °C in 5% nonfat dry milk TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) at 1:1000. Secondary horseradish peroxidase conjugated goat antirabbit IgG (Sigma) was added at 1:10,000 and incubated for 1 h at room temperature on a platform shaker followed by five washes with TBS-T. Chemiluminescence signal detection was performed with SuperSignal West Dura chemiluminescent substrate (Thermo Scientific) and developed using a Chemi Doc MP chemiluminescent imager (Bio-Rad).

For antibody sensitivity assays, purified recombinant α-SNAP was serially fivefold diluted to concentrations of 800, 160, and 32 pg. The proteins were then loaded onto an SDS–PAGE gel and immunoblots performed as above. To confirm loading of gel lanes, the gel was stained using ProteoSilver Kit (Sigma) according to manufacturer’s instruction.
2.11 Recombinant proteins

A vector encoding the ORF of the *G. soja* ortholog of *Glyma.18G02250* from PI 507623 was generated, and recombinant protein expression and purification were performed as in Bayless et al. (2016). In brief, the ORF was cloned into the pRham-N-His-SUMO expression vector, protein was purified using PerfectPro Ni-NTA resin as per manufacturer’s instructions, His and SUMO tags were cleaved by incubating the dialyzed protein with 1 unit per 100 μg protein of SUMO express protease (Lucigen) and removed by rebinding to the Ni-NTA column, and the recombinant protein was eluted in SNAP freeze-down buffer (50 mM Tris, 50 mM NaCl, 10% (w/v) glycerol, 0.5 mM TCEP, pH = 8). Both purity and quantity of protein were evaluated by Coomassie blue staining relative to BSA standards.

2.12 In vitro binding assays

In vitro binding assays were performed as previously described (Bayless et al., 2016). In brief, 20 μg of the specified recombinant α-SNAP was added to the bottom of a 1.5 mL polypropylene tube, unbound α-SNAP was removed with wash buffer, and 20 μg of either NSF or NSF<sub>RAN07</sub> in NSF binding buffer (20 mM HEPES, 2 mM EDTA, 100 mM KCl, 500 μM ATP, 1 mM DTT, 1% (w/v) PEG 4000) was added and incubated on ice for 10 min. The solution was then removed and excess NSF was removed by washing twice with NSF binding buffer. Samples were subsequently boiled in 1× SDS loading buffer and separated on a 10% Bis-Tris SDS–PAGE gel. To detect bound NSF, the gel was stained using the ProteoSilver Kit (Sigma) according to manufacturer’s instructions and quantified relative to α-SNAP abundance by densitometric analysis using ImageJ.

2.13 Nematode resistance tests

The nematode resistance tests were performed according to Niblack et al. (2009) with modifications described by Yu et al. (2016). In brief, the genotypes LD10-10198 and PI 507623 were crossed in a greenhouse. The F<sub>1</sub> seeds were generated and grown to maturity to produce an F<sub>2</sub> population. Ninety-six F<sub>2</sub> plants from the cross were evaluated in a resistance bioassay.

In the first SCN bioassay, seed of the SCN indicator lines (Niblack et al., 2009), the *G. soja* accession, and the susceptible check ‘Lee 74’ were germinated on germination paper. After 3 d, individual seedlings were transplanted into PVC tubes filled with steam-sterilized sandy soil and packed in plastic crocks. Each tube was infested with ~2,000 SCN eggs derived from either HG type 0 or HG type 2.5.7 nematode populations. The experimental unit was a tube with an individual plant and the tubes were randomized using a completely randomized design. The test with each isolate was done separately and there were three replicates of the indicator lines and six replicates of the PI. After 30 d, cysts were collected by washing roots over a 250 μm sieve. The cysts were then counted, and the female index (FI) was determined using the following equation: FI (%) = (number of female cysts on an entry) / (average number of female cysts on susceptible control ‘Lee 74’) × 100. The F<sub>2</sub> population was evaluated for resistance to a HG type 2.5.7 population according to the procedures listed above. In this test, each F<sub>2</sub> plant was placed in a separate tube and 10 plants of the susceptible control Lee 74 was included. DNA was isolated from a leaf sample taken from each F<sub>2</sub> plant according to Yu et al. (2016) and tested with the genetic marker Satt309 according to Kim et al. (2010). A single factor analysis of variance was completed to test for an association between the genetic marker and FI.

3 RESULTS

3.1 A small number of Glycine soja accessions contain a multicopy Rhg1 locus

As an entry point to identify *G. soja* accessions that potentially carry resistance alleles at *Rhg1*, we searched for accessions that carry genetic markers associated with the *G. max* *NSF<sub>RAN07</sub>* allele at *Glyma.07G195900*. *NSF<sub>RAN07</sub>* is essential for the viability of soybean plants carrying the cyst nematode resistance *Rhg1* haplotypes *rhg1-a* and *rhg1-b* (Bayless et al., 2018). The SoySNP50K Infinium BeadChip had previously been used to generate genetic data for over 19,000 domesticated soybean and over 1,000 wild soybean accessions from 84 countries, testing over 47,000 SNP loci in each accession (Song et al., 2013, 2015). In the absence of strongly predictive *Rhg1* markers in the SoySNP50K dataset, we used SNP ss715597431 that is specific for the R<sub>4</sub>Q polymorphism in the encoded NSF<sub>RAN07</sub> protein that was recently shown to be necessary for the viability of *G. max* that carry multicopy *Rhg1* haplotypes (Bayless et al., 2018). When we queried the SoySNP50K dataset for accessions annotated as *G. soja* that contain this SNP, only 21 lines had the corresponding polymorphism, representing ~1.7% of the ~1,100 *G. soja* annotated accessions (Supplemental Table S1). We hypothesized that some of these accessions might also contain *Rhg1* haplotypes that confer SCN resistance.

The *Rhg1* locus was then examined in the 21 selected *G. soja* accessions. Prior work has indicated that *Rhg1*-mediated resistance in *G. max* is driven substantially by the presence of three to 10 tandem duplicate copies of the >30 kb *Rhg1* locus, in so-called LC (*rhg1-a*) and HC (*rhg1-b*) genotypes (Cook et al., 2012, 2014; Lee et al., 2015b). Lee et al. (2015b)
noted a G. soja accession (W06/Jidong5) with three copies of the Rhg1 locus. The vast majority of soybean accessions in the USDA collection, including the cultivar Williams 82, are SCN-susceptible and carry the single-copy Rhg1 haplotype (Bayless et al., 2019; Cook et al., 2012; Lee et al., 2015b). To test the hypothesis that the RgQ-containing G. soja also carry multicyclo Rhg1 loci, we developed Rhl loci. The majority of these loci are adjacent to the 5′ terminus of the next Rhl repeat junction, which should only give a product if the 3′ terminus of one Rhl repeat segment is adjacent to the 5′ terminus of the next Rhl repeat. Screening of the 21 G. soja accessions revealed that six contain a duplicated Rhl (Figure 1b). Use of a different oligonucleotide pair, and a limited set of changes to the template preparation and PCR conditions, produced the same results regarding presence of the repeat junction PCR product in these accessions (Supplemental Figure S1a).

### 3.2 The Glycine soja multicyclo Rhg1 locus shares structural characteristics with Glycine max multicyclo Rhg1 haplotypes

The Rhl repeat junction was previously shown to be conserved between HC and LC SCN-resistant G. max genotypes, suggesting a shared lineage (Cook et al., 2012, 2014; Lee et al., 2015b). To test whether these multicyclic Rhl G. soja loci also share that lineage, we cloned and sequenced the repeat junction from the six repeat-positive genotypes shown in Figure 1b. Those G. soja accessions were found to carry a Rhl repeat junction that is identical to that of the Rhl-a soybean genotype Peking and the Rhl-b soybean accession PI 88788 (Figure 1c). As previously reported, the Rhl repeat junction is not present in the single-copy Rhl of the G. max reference genome for Williams 82 (Figure 1c). The above observations support the hypothesis that all known multicyclic Rhl types in annual Glycine species arose from a shared event. Because of the differences from G. max multicyclic Rhl haplotypes we describe later, and to follow upon established use of the allele terms rhl1-a, rhl1-b, and rhl1-c for previously described soybean haplotypes, we named this G. soja multicyclic Rhl haplotype ‘rhl1-ds’, short for rhl1-d (soja).

As it is possible that G. soja accessions carrying the rhl1-ds haplotype arose through hybridization events, we performed unrooted phylogenetic analyses (Huson & Bryant, 2006). Phylogenetic analyses were first conducted using genome-wide SNP data from the SoySNP50K dataset (Song et al., 2013, 2015), to assess the overall relatedness of accessions carrying NSF_RAN07 and rhl1-ds, to each other and to a more broadly representative set of G. soja accessions. The comparison set was a relatively random set of G. soja accessions chosen because they have been included in recent publications from other research groups (Figure 2a; Supplemental Figure S2a). At the whole-genome scale, G. soja accessions carrying an NSF_RAN07-encoding allele are dispersed throughout the phylogeny (Figure 2a). The rare rhl1-ds-containing G. soja accessions were more narrowly clustered, suggesting a shared derivation within G. soja. Unsurprisingly, individual rhl1-ds-containing G. soja also can be closely related to G. soja that do not carry rhl1-ds (Figure 2a). At a genome-wide level the rhl1-ds-containing G. soja group separates from G. max that carry rhl1-a or rhl1-b, suggesting that rhl1-ds arose independent of these haplotypes rather than from a recent hybridization with G. max that carry rhl1-a or rhl1-b (Supplemental Figures S2a and S2b).

To study the relatedness of the rhl1-ds locus to the rhl1-a and rhl1-b loci, we used a targeted approach to extract and sequence genomic DNA from Rhl resulting in >70% coverage of the rhl1-ds loci from PI 507613, PI 407287, and PI 378695A. These accessions were chosen because they are relatively distant from each other in the phylogenetic analysis (Figure 2a). The data were compared with previously determined Rhl sequences from G. max and G. soja. Figure 2b shows that rhl1-ds forms a clade with rhl1-a and rhl1-b that is distinct from the more common wild-type (single-copy, SCN-susceptible) haplotypes. The Rhl loci from the susceptible G. max and G. soja are more similar to each other than to rhl1-a, rhl1-b, or rhl1-ds. Although the shared origin of soybean rhl1-a and rhl1-b and G. soja rhl1-ds was already implied from their identical Rhl repeat junction sequences (Figure 1c), the phylogeny shown in Figure 2b further demonstrates their apparent shared derivation. The data also suggest that rhl1-ds arose prior to the split between rhl1-a and rhl1-b.

We selected two rhl1-ds G. soja accessions, PI 507613 and PI 507623 (Figure 2a), to characterize in further detail. They were collected in 1983 as wild plants, ~80 km from each other, in central Japan. We also continued to study rhl1-ds-containing PI 342434 because, although presently annotated in the USDA germplasm collection as a G. max, it carries leaf shape and plant architecture traits that are intermediate between G. max and G. soja and contains a genome-wide SNP pattern that clusters with G. soja accessions (Figure 2a). PI 342434 is also from Japan (donated to USDA in 1969) and was originally annotated as a Glycine ursuensis (Regel & Maack) ‘tsurumame’ edible wild soybean. We sought to determine the Rhl copy number of these three accessions using both a genomic DNA qPCR method and a copy number variation TaqMan assay as in Lee et al. (2015b, 2016). Our implementation of the TaqMan assay (example shown in...
Supplemental Figure S1b) was unsuccessful, as we obtained erratic data even for controls, but those assays did indicate a rhg1-ds copy much lower than in rhg1-b cultivar Fayette and similar to that of rhg1-a cultivar Forrest. Genomic qPCR assays (Figure 1d) were more reproducible and also included domesticated soybean cultivars of known Rhg1 copy number 1, 3, and 10 as controls. The tested G. soja and G. max had approximately the same number of copies of Rhg1 as Peking (three copies; Figure 1d). This is consistent with the hypothesis that after multicopy Rhg1 haplotypes became established, higher soybean Rhg1 copy number soybeans such as rhg1-b with nine or 10 copies may have been a trait derived under positive selection during breeding (Lee et al., 2015b).

3.3 Glycine soja multicopy Rhg1 encodes a distinct α-SNAP

Investigation of the G. soja rhg1-ds locus revealed that it encodes a novel Rhg1 α-SNAP variant (Figure 3a). The contributions of Rhg1 α-SNAP proteins to SCN resistance depend heavily on the presence of altered sets of C-terminal amino acid residues at sites that are otherwise highly conserved across multicellular eukaryotes (Bayless et al., 2016; Cook et al., 2014). Distinct sets of C-terminal amino acids are carried by each of the Glyma.18G022500 Rhg1 protein products: α-SNAPRhg1-LC (from LC, Peking-type rhg1-a) and α-SNAPRhg1-HC (from HC, PI 88788-type rhg1-b) (Cook et al., 2014; Figure 3a). The multicopy G. soja rhg1-ds haplotypes and the product from G. max PI 342434 encoded an identical α-SNAP that diverged from the susceptible G. max and G. soja reference genomes (wild-type Wm82 and G. soja W05, and PI 483463, respectively), and from α-SNAPRhg1-LC and α-SNAPRhg1-HC (Figure 3a). To differentiate this protein from the much more common wild-type α-SNAPRhg1 protein that is produced in G. soja accessions that carry single-copy Rhg1 haplotypes, and in adherence with the original naming convention for Rhg1-encoded α-SNAP proteins (Bayless et al., 2016), we termed this protein ‘α-SNAPRhg1-Gsm’, for G. soja multicity. The single-copy Rhg1 G. soja accessions studied because they carried the NSF_RAN07 R4Q polymorphism were all found to encode an α-SNAP
α-SNAP$_{\text{Wm82}}$ (WT) KAKELEEDD–LT
α-SNAP$_{\text{G. soja}}$ (WT) KAKELEEDD–LT
α-SNAP$_{\text{G. soja} \mathrm{HC} (\text{rhg1-b})}$ KAKELEQHEAIT
α-SNAP$_{\text{G. soja Gsm (rhg1-ds)}}$ KAKELEEIQEVIT
α-SNAP$_{\text{G. soja HC} (\text{rhg1-h})}$ KAKELEEIQEVIT

**FIGURE 3** Unique *Glycine soja* alpha-Soluble NSF Attachment Protein (α-SNAP) induces cell death and N-ethylmaleimide Sensitive Factor (NSF) hyperaccumulation in *Nicotiana benthamiana*. (a) Alignment of C-terminal amino acids of the *Resistance to Heterodera glycines* I (*Rhg1*) α-SNAP from multicopy *rhg1-ds* *Glycine soja* (α-SNAP$_{\text{Rhg1 Gsm}}$) compared with *Rhg1* α-SNAPs from single-copy SCN-susceptible *G. soja* W82 (Wm82; wild type [WT]) or *G. soja* W05 (*G. soja*; WT), and multicopy SCN-resistant *G. max* cultivars. PI 88788 (high copy [HC]; *rhg1-b*) and Peking (low copy [LC]; *rhg1-a*). (b) Representative *N. benthamiana* leaf 7 d after infiltration with *A. tumefaciens* expressing empty vector negative control or the denoted *Rhg1* α-SNAP with no epitope tag [abbreviations as in (a)]. (c) Immunoblots detecting levels of endogenous *N. benthamiana* NSF or α-SNAP (α-SNAP-WT), as well as recombinant *Glycine* α-SNAPs delivered via *A. tumefaciens* as in (b). Same sample was used for entire columns in (c) but probed with three separate antibodies. Leaf tissue was harvested ~60 h after infiltration; Ponceau S staining indicates relative levels of total protein in each sample.

3.4 | *Glycine soja Rhg1* α-SNAP interacts weakly with wild-type NSF$_{Ch07}$

In light of the above results, we investigated the interaction between α-SNAP$_{\text{Rhg1 Gsm}}$ and the two NSF types, wild-type NSF$_{Ch07}$ and *G. soja*-encoded NSF$_{\text{RAN07}}$. We first
validated the presence of NSF\textsubscript{RAN07} in the six \textit{G. soja} \textit{rhg1-ds} accessions using primers specific either to the NSF\textsubscript{RAN07} or NSF\textsubscript{Ch07} and NSF\textsubscript{Ch13} (\textit{Glyma}.13G180100) as a positive control. As predicted by the initial SoySNP 50K screen of \textit{G. soja} germplasm, all six of the tested accessions had NSF\textsubscript{RAN07} amplicons (Supplemental Figure S3). Interestingly, PI 507614B and PI 407287 gave amplicons for both NSF\textsubscript{RAN07} and NSF\textsubscript{Ch07} across plant samples, whereas the other accessions only gave amplicons to NSF\textsubscript{RAN07} (Supplemental Figure S3). Subsequent sequencing of PCR products from PI 507614B or PI 407287, generated using primers specific to the 5’ portion of \textit{Glyma}.07G195900 (5’ UTR and first intron), only showed sequences analogous to NSF\textsubscript{RAN07}. This suggests that an additional NSF\textsubscript{Ch07} like locus may be adjacent to sequences in the genome not captured by our primer set. Cloning of \textit{Glyma}.07G195900 for each of the six \textit{rhg1-ds} \textit{G. soja} accessions as well as PI 342434 \textit{G. max} revealed a DNA sequence identical to that of the NSF\textsubscript{RAN07} from known resistant-type soybeans. Sequencing of a PCR product carrying the 5’ portion of PI 468396B, previously characterized by from Zhang et al. (2017a), which does not have the R\textsubscript{Q} polymorphism-associated SNP, showed an NSF\textsubscript{Ch07} allele. This further indicates the accuracy of the above primer set. We subsequently determined the sequence of the NSF\textsubscript{Ch07} amplicons in PI 507614B and 407287, by cloning the amplicon into a pGEM-T plasmid and sequencing. Intronic polymorphisms revealed that the amplicons derived from PI 507614B and PI 407287 have a sequence similar to the NSF\textsubscript{Ch07} from Williams 82. This suggests that there is an NSF\textsubscript{Ch07} allele in these accessions, although perhaps at a chromosomal location that was not amplified from the above primer set.

We then proceeded to investigate physical interaction between \textalpha-SNAP\textsubscript{Rhg1} \textit{Gsm} protein and the two NSF types. Using an \textit{Escherichia coli} expression system, we produced recombinant \textalpha-SNAP\textsubscript{Rhg1} \textit{Gsm} protein and NSF\textsubscript{Ch07} and NSF\textsubscript{RAN07} proteins, as well as \textalpha-SNAP\textsubscript{Rhg1} \textit{LC} and \textalpha-SNAP\textsubscript{Rhg1} \textit{HC} from resistant \textit{G. max} varieties, and \textalpha-SNAP\textsubscript{Rhg1} \textit{WT} from single-copy susceptible Williams 82. We then performed in vitro binding assays between the different NSFs and \textalpha-SNAPs. Relative to single-copy susceptible Williams 82, the resistance-associated \textit{G. max} \textalpha-SNAPs interacted poorly with wild-type NSF\textsubscript{Ch07} (Figure 4a), recapitulating prior observations (Bayless et al., 2016, 2018). The \textit{G. soja} \textalpha-SNAP\textsubscript{Rhg1} \textit{Gsm} also exhibited deficient interaction with wild-type NSF\textsubscript{Ch07} (Figure 4a). This suggests that, as occurs with the resistance-associated soybean \textit{Rhgl} \textalpha-SNAPs, presence of the \textit{G. soja} \textalpha-SNAP\textsubscript{Rhg1} \textit{Gsm} is likely to disrupt the normal cellular vesicle trafficking that requires efficient \textalpha-SNAP–NSF interaction in the absence of NSF\textsubscript{RAN07}. These results are quantified in Figure 4b. We also investigated if \textalpha-SNAP\textsubscript{Rhg1} \textit{Gsm} interacts better with NSF\textsubscript{RAN07} than with NSF\textsubscript{Ch07}. Consistent with previous findings regarding \textalpha-SNAP\textsubscript{Rhg1} \textit{LC} and \textalpha-SNAP\textsubscript{Rhg1} \textit{HC} (Bayless et al., 2018),

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{\textit{Glycine soja} \textit{rhg1-ds} alpha-Soluble NSF Attachment Protein (\textalpha-SNAP\textsubscript{Rhg1} \textit{Gsm}) interacts well with NSF\textsubscript{RAN07} N-ethylmaleimide Sensitive Factor protein but poorly with wild-type NSF. (a) Representative silver-stained SDS/PAGE gel of recombinant soybean wild-type (WT) NSF\textsubscript{Ch07} or NSF\textsubscript{RAN07} bound in vitro by different recombinant \textalpha-SNAP\textsubscript{Rhg1} proteins in relative binding affinity assay. LC, \textalpha-SNAP\textsubscript{Rhg1} \textit{LC} (low copy); Gsm, \textalpha-SNAP\textsubscript{Rhg1} \textit{Gsm}; HC, \textalpha-SNAP\textsubscript{Rhg1} \textit{HC} (high copy); WT, Wild type (single-copy) \textalpha-SNAP\textsubscript{Rhg1}; BSA, bovine serum albumin (carrier protein). (b) Densitometric quantification of relative amount of NSF\textsubscript{Ch07} (upper graph) or NSF\textsubscript{RAN07} (lower graph) bound by the indicated \textalpha-SNAP, collecting results from three independent experiments. Error bars show standard error of the mean. Bars with same letter are not statistically different (ANOVA, Tukey HSD \(p > .05\))}
\end{figure}
there was marked increase in the binding of $\alpha$-SNAP$_{Rhg1}$Gsm to NSF$_{RAN07}$ relative to binding with wild-type NSF$_{Ch07}$ (Figures 4a and 4b).

### 3.5 Glycine soja with multicyclic rhgl-ds do not carry the rhgl-α α-SNAP copia retrotransposon, chromosome 11 α-SNAP truncation, or resistance-associated Rhg4

The additional genetic features known to most prominently associate with some Rhgl-mediated SCN resistance are the rhgl-α α-SNAP copia (RAC) retrotransposon, the chromosome 11 α-SNAP intron-retention allele that encodes a non-functional truncated α-SNAP, and resistance-associated alleles of Rhg4 that encode a serine hydroxymethyltransferase (Bayless et al., 2018, 2019; Lakhssassi et al., 2017; Liu et al., 2012). We examined the six rhgl-ds G. soja accessions (and PI 342434 G. max) for these genetic features. None of these seven accessions had a SNP signature of RAC integration (ss71560985 G to A; Supplemental Table S1) or any PCR-amplification features of the 4.8 kb RAC insertion at Glyma.18G022500 (Figure S4b). They also did not carry short sequence repeats that could be a hallmark of transposon excision at a possible excised RAC site in rhgl-ds.

The genomic region that in some soybean accessions encodes an intron-retention allele at the Glyma.11G234500 α-SNAP$_{Ch11}$ gene was also sequenced. We first examined SoySNP50K data for the SNP associated with the intron retention allele ss71560985 C to T but noted that for many G. soja the nucleotide was uncalled at this SNP position. By PCR and sequencing, we determined that all seven of the rhgl-ds accessions carried an allele lacking the intron retention SNP (Supplemental Figure S5). When antibodies against α-SNAP$_{WT}$ were used in protein immunoblots, we discovered that PI 507613, but not PI 507623, had a low apparent level of α-SNAP$_{WT}$ protein in both roots and shoots (Figure 5a; Supplemental Figure S6a). This low level of α-SNAP$_{WT}$ is analogous to previous experiments with rhgl-a soybean lines that carry the α-SNAP intron-retention allele on chromosome 11 (Bayless et al., 2018). DNA sequencing revealed that while the encoded C-terminus of α-SNAP$_{Ch11}$ in PI 507623 is similar to Williams82, the C-terminus of α-SNAP$_{Ch11}$ in PI 507613 carries a single amino acid polymorphism (E$_{285}$V; Figure 5b). The apparent low levels of α-SNAP$_{WT}$ protein in PI 507613 may be due to reduced recognition by the antibody used, or to other regulatory mechanisms, but apparently are not due to a Glyma.11G234500 intron retention allele.

We also performed immunoblots with leaf and root extracts of PI 507613 and PI 507623 using other antibodies to detect resistance-associated α-SNAP$_{Rhg1}$LC and α-SNAP$_{Rhg1}$HC. For tissue from developing trifoliates, immunoblots revealed a low basal expression of α-SNAP$_{Rhg1}$Gsm in G. soja PI 507613 and PI 507623 relative to the Forrest (rhgl-a) control (Supplemental Figure S6a). However, in rhgl-ds roots we observed more α-SNAP$_{Rhg1}$Gsm protein, at levels analogous to Forrest soybean roots (Figure 5a). Control blots of purified α-SNAP$_{Rhg1}$Gsm protein showed antibody recognition at sensitivities similar to that for α-SNAP$_{Rhg1}$HC or LC, against which the antibodies were raised (Supplemental Figure S6b).

Rhg4 encodes a variant serine hydroxymethyltransferase in the allele (not genetically linked to Rhg1) that plant breeders pair with rhgl-a to achieve useful SCN resistance in domesticated soybean (Liu et al., 2012; Patel et al., 2019; Yu et al., 2016). Sequencing of Rhg4 cDNA clones from the six rhgl-ds G. soja accessions (as well as soybean Rhg4 transcripts from SCN-susceptible cultivar Essex and SCN-resistant Peking as controls) revealed absence of the P$_{130}$R and N$_{358}$Y polymorphisms associated with Peking-type resistance, suggesting that the six rhgl-ds G. soja accessions carry a susceptible-type (wild-type) Rhg4.

### 3.6 The Glycine soja PI 507613 and PI 507623 are resistant to virulent nematode populations

In light of the above observations about rhgl-ds, including observations about the absence of a resistance-associated
Rhg4 and chromosome 11 α-SNAP alleles, we sought to determine the SCN resistance of select rhg1-ds G. soja accessions. The rhg1-ds G. soja lines PI 507613, PI 507623, and the G. soja-like PI 342434 were tested with HG type 0 and HG type 2.5.7 nematode populations. We compared the FIs (relative number of cysts formed) to those for G. max HG type indicator lines and the G. soja PI 468916, which is the source of the SCN resistance QTL cqSCN-006 and cqSCN-007 (Wang et al., 2001) (Table 1). As might be predicted because of the absence of resistance-associated Rhg4 or chromosome 11 α-SNAP alleles, PI 342434 was only able to confer moderate resistance to an HG type 0 SCN population. Intriguingly, PI 342434 was not able to confer substantial resistance to an HG type 2.5.7 SCN population. Even more intriguing, PI 507613 and PI 507623 were both able to confer strong resistance to HG type 0 and HG type 2.5.7 nematode populations, with levels comparable to PI 468916 (Table 1). The above findings, taken as a whole, do not demonstrate causation but do suggest that the novel rhg1-ds haplotype is as functional as rhg1-a for SCN resistance against HG type 0 SCN. The resistance of PI 507613 and PI 507623 to the HG type 2.5.7 SCN population (that PI 342434 was susceptible to) indicates that in PI 507613 and PI 507623, loci other than rhg1-ds also contribute to SCN resistance against economically important HG type 2.5.7 SCN populations. A summary of these data may be found in Supplemental Table S2.

### TABLE 1

| HG type indicator line number | Accession | HG Type 0 | HG Type 2.5.7 |
|-----------------------------|-----------|----------|---------------|
| 1                           | ‘Peking’ | 0        | 0             |
| 2                           | PI 88788 | 1        | 42            |
| 3                           | PI 90763 | 0        | 0             |
| 4                           | PI 437654 | 0      | 0             |
| 5                           | PI 209332 | 1    | 47            |
| 6                           | PI 89772 | 0        | 0             |
| 7                           | ‘Cloud’ | 3        | 62            |
| –                           | PI 507623 | 9    | 8             |
| –                           | PI 507613 | 3    | 14            |
| –                           | PI 342434 | 26   | 60            |
| –                           | PI 468916 | 2    | 18            |

*Female index is percentage of SCN cysts per root system, relative to the susceptible control (‘Lee 74’) within the same experiment, for 10 plants per genotype.

### 3.7 rhg1-b and rhg1-ds made statistically similar partial-resistance contributions against an HG type 2.5.7 SCN population

To test the effect of the novel rhg1-ds allele on resistance relative to the rhg1-b allele, a F2 population from a cross of G. max LD10-10198 (rhg1-b resistance) × G. soja PI 507623 (rhg1-ds) was tested for SCN resistance. Each plant in the population was evaluated for resistance in a greenhouse using an HG type 2.5.7 SCN population against which rhg1-b soybean exhibit partial resistance, and each plant was tested with the marker Satt309 that maps within 1 cM of Rhg1 (Kim et al., 2010). One month after the plants were inoculated, the susceptible check Lee 74 averaged 219 SCN females, while the 96 F2 plants averaged 120 females. Within the F2 population, no significant association was observed between the Satt309 allele present and level of SCN resistance [single factor ANOVA Pr(>F) = 0.34]. Hence rhg1-ds was associated with partial resistance—no distinction in their contribution to resistance against the tested HG type 2.5.7 SCN population was detected between rhg1-b and the rhg1-ds haplotype derived from PI 507623. This experiment, together with that of Table 1 (above), also provides further evidence that there are loci other than Rhg1 in PI 507623 that contribute to the observed high-level resistance of PI 507623 to the virulent HG type 2.5.7 SCN population that was used.

### 4 DISCUSSION

Crop wild relatives are an appealing source for novel traits of agronomic interest, and this is evident in domesticated soybean. Recent resequencing of G. soja along with landrace and elite G. max varieties revealed that many rare alleles were lost from G. soja during its domestication to form G. max (Hyten et al., 2006; Liu et al., 2020). Many of these lost traits are of agronomic importance. Quantitative trait loci associated with improved yield, salt tolerance, alkaline salt tolerance, soybean aphid resistance, foxglove aphid resistance, and SCN resistance have all been found to be present in G. soja but absent in G. max (Concibido et al., 2003; Hesler et al., 2013; Kabelka et al., 2005, 2006; Kim et al., 2013; Lee et al., 2009, 2015a; Wang et al., 2001; Yu & Diers, 2017). With respect to SCN, G. soja PI 468916 has served as a donor for the QTL cqSCN-006 and cqSCN-007 that each contribute modest resistance to a highly virulent SCN population but provide substantial and synergistic resistance even to HG type 1.2.3.5.6.7 SCN when combined in G. max with rhg1-b and a chromosome 10 QTL from PI 567516C (Brzostowski et al., 2017). Here, we used a SNP marker associated with NSF_RAN07 to select G. soja accessions to screen for multicopy Rhg1 haplotypes, leading to discovery of the rhg1-ds haplotype that encodes a unique
α-SNAP with SCN resistance-associated functional features. The hypothesis was that plant germplasm accessions that are strong candidates to carry alleles of interest can be identified using available SNP genotypes for particular alleles of other (unlinked) genes that also contribute to the trait of interest. Our study highlights the power of deploying new findings regarding the genetic architecture of traits of interest (such as the requirement for NSF\textsubscript{RAN07} in Rhg1-mediated SCN resistance), along with available data such as the SoySNP50K data for ∼20,000 Glycine accessions, to prescreen germplasm and identify a manageable number of accessions for functional analysis. Recent next-generation sequencing work has further identified structural and nucleotide variants within domesticated soybean (Torkamaneh et al., 2019). The same study also identified loss of function alleles within soybean germplasm, potentially functioning as a sequence-catalogued mutant library analogous to those available in Arabidopsis thaliana (L.) Heynh. If genetic features of potential interest are known, there are increasingly powerful resources available for in silico germplasm prescreens.

A potential limitation of using the NSF\textsubscript{RAN07} SNP for in silico germplasm screens is that it may not fully capture the diversity of Rhg1-containing G. soja. There may be lines that do not share with soybean (Bayless et al., 2018) the requirement for NSF\textsubscript{RAN07} for Rhg1-mediated SCN resistance. In the present study and in previous work, NSF\textsubscript{RAN07} was found to be present in all multicopy Rhg1 accessions but also in a minority of the much larger pool of single-copy Rhg1 accessions (Bayless et al., 2018), suggesting that NSF\textsubscript{RAN07} is necessary for multicopy Rhg1 resistance while demonstrating that NSF\textsubscript{RAN07} can be present in the absence of multicopy Rhg1 resistance. Some accessions expressing NSF\textsubscript{RAN07} but lacking multicopy Rhg1 might exhibit elevated SCN resistance, but that trend has not been observed in the documented SCN resistance of soybean accessions that express NSF\textsubscript{RAN07} but lack multicopy Rhg1.

We and others have identified SoySNP 50K (or KASP) SNPs associated with other resistance-associated-genetic elements that might be used during in silico germplasm screens for possible SCN resistance. In addition to NSF\textsubscript{RAN07}, these include the chromosome 11 intron retention α-SNAP knockout, the RAC (rhg1-a-alpha SNAP copia) retrotransposon in the first intron of rhg1-a α-SNAP genes, and SNPs specific for SCN resistance-associated alleles of Rhg4 (Bayless et al., 2018, 2019; Matsye et al., 2012; Shi et al., 2015; Tran et al., 2019). These might be used to select G. soja accessions that can subsequently be tested for the presence of useful alleles at other loci that contribute to SCN resistance whose presence is not adequately tested by the SoySNP50K dataset. More direct tests using markers not on the SoySNP50K chip could also be carried out de novo on large numbers of germplasm accessions (albeit at appreciable new expense), for example to detect Rhg1 tandem repeat junctions, novel C-terminal sequences in α-SNAP proteins, or new Rhg4 alleles.

The present discovery of PI 507613 as an accession of interest for SCN resistance provides an extended example of the above hypothesis, that useful alleles at multiple loci controlling a trait are likely to co-occur in individual accessions. Phenotypic screening of USDA accessions for SCN resistance apparently missed PI 507613, which was instead flagged for further study by our NSF\textsubscript{RAN07} genotypic screen. PI 507613 would have been passed over in a screen for RAC but would be positive in a screen for the Rhg1 tandem repeat junction. PI 507613 would also be negative for Rhg4 or the chromosome 11 intron-retention α-SNAP, but we did discover another α-SNAP with unusual C-terminal amino acids in this accession. The example gains more interest when considering that, in addition to finding the novel rhg1-a, we found that PIS07613 carries the valuable trait of SCN resistance to HG type 2.5.7 SCN (that apparently is not dependent on traditional rhg1-α/Rhg4/chromosome 11 α-SNAP genotypes). This PI 507613 SCN resistance is dependent on more loci than just rhg1-a. We postulate that it was much more likely to identify such an accession once we focused on G. soja accessions that carry one or more SCN resistance alleles of interest. A productive target for future studies will be to map and identify the full complement of other loci that contribute to resistance to HG type 2.5.7 SCN in PI 507613, PI 507623, and possibly in the other rhg1-a PIs identified in this study.

Unlike domesticated soybean, G. soja accessions are not known to contain the resistance-associated allele of Rhg4, which is necessary for the efficacy of SCN resistance conferred by rhg1-a in G. max (Brucker et al., 2005; Meksem et al., 2001; Wu et al., 2016; Yu et al., 2016). Our findings are consistent in these observations. The accessions we examined did not have a resistance-type Rhg4 or other attributes of Peking-type SCN resistance: presence of RAC or the nonfunctional chromosome 11 α-SNAP allele (although in PI 507613 there does seem to be a novel α-SNAP\textsubscript{Ch11}). Previous GWAS studies with G. soja have either not detected significant contributions from the Rhg1 locus when testing with a Race 1 (HG type 2.5.7) SCN population (Zhang et al., 2017a) or detected only a minor contribution ($R^2 = 13.6\%$) from a locus 5–12 Mb (>8 genes) away from Rhg1 when testing with a separate HG type 2.5.7 SCN population (Zhang et al., 2016). Those GWAS results may have been impacted by a very low rate of occurrence of multicopy Rhg1 loci in G. soja. However, they are also consistent with poor expression of the SCN resistance phenotype that might be predicted if a haplotype such as rhg1-a is not coupled with complementary Rhg4/RAC/chr11 α-SNAP genotypes. This makes it all the more interesting to understand the other genetic features that contribute to any observed SCN resistance in G. soja (possibly including loci identified by Kabelka et al. [2005], Winter et al. [2007], Arelli et al. [2000], Vuong et al. [2015], Zhang et al. [2016, 2017a,
The evolutionary history of the economically important *rhgl-b* and *rhgl-a* soybean loci is a point of interest, and not only because of the unique structure of these loci (copy number variation of up to 10 copies of a four gene chromosomal block, in which three of the four tightly linked genes contribute to SCN resistance). Investigations of *Rhgl* evolution may also reveal new *Rhgl* haplotypes or suggest approaches to additional functional engineering of *Rhgl* components. The previous cloning and characterization of both *rhgl-a* and *rhgl-b* demonstrated that the bridge junction (the junction between two *Rhgl* blocks) is conserved (Cook et al., 2012). This suggested that *Rhgl* duplication preceded the split between *rhgl-a* and *rhgl-b*. Further studies have revealed an array of copy numbers for *rhgl-a* and *rhgl-b* haplotypes in various soybean accessions (Cook et al., 2012, 2014; Lee et al., 2015b, 2016; Patil et al., 2019; Yu et al., 2016). Whole-genome resequencing has suggested the existence of a *rhgl-a*-like *Rhgl* within *G. soja*, providing evidence for an early origin of *Rhgl* outside of *G. max* (Lee et al., 2015b). The data we present provides more complete evidence of an early origin of multicopy *Rhgl*, and revealed a novel haplotype and novel α- SNAP*Rhgl*. Further, this study identified *rhgl-ds* as a candidate progenitor of *rhgl-a* and *rhgl-b* and α- SNAP*Rhgl*Gsm as a candidate progenitor of α- SNAP*Rhgl*LC and α- SNAP*Rhgl*HC. The multiyear undertaking has been initiated to generate transgenic soybean lines in which isogenic presence or absence of the allele encoding α- SNAP*Rhgl*Gsm can be associated with resistance to different HG types of SCN.

Vesicle trafficking is essential to eukaryotic cellular homeostasis. The aberrant α- SNAP encoded by resistance-associated *Rhgl* haplotypes has been implicated in conferring resistance to SCN (Bayless et al., 2016; Cook et al., 2012, 2014; Lakhssassi et al., 2017, 2020; Liu et al., 2017). Interestingly, the only SNPs causing nonsynonymous codon differences in the *Rhgl* proteins from resistant and susceptible cultivars occur within the gene body of the α-SNAP, particularly at the C-terminus—the region that stimulates NSF activity for SNARE disassembly (Barnard et al., 1997; Bayless et al., 2016; Cook et al., 2014; Marz et al., 2003; Zhao et al., 2015). Here, we report an additional resistance-associated α- SNAP with a C-terminus distinct from that of either known resistant types or the standard wild-type α- SNAP. Variation in nematode functions that interface with host plant α-SNAPs might be associated with SCN virulence. Recent work elucidating effectors of SCN suggests that SNARE-like proteins—proteins that normally interact with α-SNAP and NSF to form the 20S complex—constitute a large and variable effector family in SCN (Gardner et al., 2018). Further, there is some molecular evidence for direct physical interaction between SNARE-like protein effectors and α-SNAP with virulence outcomes (Bekal et al., 2015). Moreover, recent evidence suggests a potential physical interaction between the *Rhgl*-encoded serine hydroxymethyltransferase and the α-SNAP*Rhgl* as well as α-SNAP*Rhgl* and syntaxin proteins, both with implications for resistance (Dong et al., 2020; Lakhssassi et al., 2020). In light of this, varying the soybean α-SNAP repertoire (for example by use of *rhgl-ds* and α-SNAP*Rhgl*Gsm) may hinder the capacity of nematodes to overcome *Rhgl*-mediated resistance.

The present study also discovered a novel α-SNAP on chromosome 11 in PI 507613. From previous work, it is apparent that SCN resistance involves not just presence of a α-SNAP*Rhgl* but also modification of the relative amount of wild-type α-SNAP protein in syncytia or in whole plants (Bayless et al., 2016, 2018, 2019). It is possible that the novel α-SNAP*Ch11* of PI 507613 is a bona fide resistance-associated QTL. Indeed, under a model where HG type 0 or HG type 2.5.7 nematode populations produce effectors that interact with α-SNAPWT toward facilitating infection or reproduction, modifying the α-SNAPWT could change virulence outcomes as a result of reduced interaction. Future work should be directed toward understanding whether the novel α-SNAP*Ch11* cosegregates with SCN resistance.

It may be possible to engineer soybean with novel α-SNAPs by genetic modification and gene-editing methods, or by classical breeding to bring in different α-SNAPs such as the *G. soja rhgl-ds* product. Through work such as that of Marz et al. (2003), a library of mammalian α-SNAP mutations and known consequences is already available, and this type of structure–function knowledge (see also Zhao et al., 2015) is ready to be translated to soybean α-SNAP. Vesicle trafficking has been implicated in many plant-pathogen interactions beyond SCN (Hoefle & Hückelhoven, 2008; Inada & Ueda, 2014). Because of the seeming centrality of vesicle trafficking in plant defense, α-SNAP protein sequences and expression patterns may also represent appealing targets to modify in order to confer resistance to other biotrophic pathogens.

**DATA AVAILABILITY STATEMENT**

The data supporting the conclusions of this article are included within the article and the supplementary materials.

**ACKNOWLEDGMENTS**

We thank Adam Bayless for early contributions to the study and Alison Colgrove for assistance with nematode experiments. Funding for this study was provided by United Soybean Board awards to A.B. and B.D., by the Department of Plant Pathology and the Agricultural Experiment Station Hatch program at University of Wisconsin–Madison, and by National Science Foundation Predoctoral Fellowship and NIH Molecular Biosciences Training Grant awards to D.G.
AUTHOR CONTRIBUTIONS
Derrick G. Grunwald: Conceptualization; Investigation; Writing-original draft; Writing-review & editing. Ryan W. Zapotocny: Conceptualization; Investigation; Writing-review & editing. Seda Ozer: Investigation; Writing-review & editing. Brian Diers: Funding acquisition; Investigation; Writing-review & editing. Andrew Bent: Conceptualization; Funding acquisition; Investigation; Writing-original draft; Writing-review & editing.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

ORCID
Brian W. Diers https://orcid.org/0000-0003-3584-5495
Andrew F. Bent https://orcid.org/0000-0001-6610-9525

REFERENCES
Arelli, P. R., Sleper, D. A., Yue, P., & Wilcox, J. A. (2000). Soybean reaction to Races 1 and 2 of Heterodera glycines. Crop Science, 40, 824–826. https://doi.org/10.2135/cropsci2000.403824
Barnard, R. J. O., Morgan, A., & Burgoyne, R. D. (1997). Stimulation of NSF ATPase activity by α-SNAP is required for SNARE complex disassembly and exocytosis. Journal of Cell Biology, 139, 875–883. https://doi.org/10.1083/jcb.139.4.875
Barszczewski, M., Chua, J. J., Stein, A., Winter, U., Heintzmann, R., Zilly, F. E., Fasshauer, D., Lang, T., & Jahn, R. (2008). A novel site of action for α-SNAP in the SNARE conformational cycle controlling membrane fusion. Molecular Biology of the Cell, 19, 776–784. https://doi.org/10.1091/mbc.e07-05-0498
Bayless, A. M., Smith, J. M., Song, J., McMinn, P. H., Teillet, A., August, B. K., & Bent, A. F. (2016). Disease resistance through impairment of α-SNAP–NSF interaction and vesicular trafficking by soybean Rhg1. Proceedings of the National Academy of Sciences, 113, E7375–E7382. https://doi.org/10.1073/pnas.1610150113
Bayless, A. M., Zapotocny, R. W., Grunwald, D. J., Amundson, K. K., Diers, B. W., & Bent, A. F. (2018). An atypical N-ethylmaleimide sensitive factor enables the viability of nematode-resistant Rhg1 soybeans. Proceedings of the National Academy of Sciences, 115, E4512–E4521. https://doi.org/10.1073/pnas.1717070115
Bayless, A. M., Zapotocny, R. W., Han, S., Grunwald, D. J., Amundson, K. K., & Bent, A. F. (2019). The rhg1-a (Rhg1 low-copy) nematode resistance source harbors a copia-family retrotransposon within the Rhg1-encoded α-SNAP gene. Plant Direct, 3, e00164. https://doi.org/10.1002/pld3.164
Bekal, S., Domier, L. L., Gonfa, B., Lakhssassi, N., Meksem, K., & Lambert, K. N. (2015). A SNARE-like protein and biotin are implicated in soybean cyst nematode virulence. PLoS ONE, 10, e0145601. https://doi.org/10.1371/journal.pone.0145601
Brucker, E., Carlson, S., Wright, E., Niblack, T., & Diers, B. (2005). Rhg1 alleles from soybean PI 437654 and PI 88788 respond differentially to isolates of Heterodera glycines in the greenhouse. Theoretical and Applied Genetics, 111, 44–49. https://doi.org/10.1007/s00122-005-1970-3
Brzostowski, L. F., & Diers, B. W. (2017). Pyramiding of alleles from multiple sources increases the resistance of soybean to highly virulent soybean cyst nematode isolates. Crop Science, 57, 2932–2941. https://doi.org/10.2135/cropsci2016.12.1007
Colgrove, A., & Niblack, T. (2008). Correlation of female indices from virulence assays on inbred lines and field populations of Heterodera glycines. The Journal of Nematology, 40, 39–45.
Concibido, V. C., Diers, B. W., & Arelli, P. R. (2004). A decade of QTL mapping for cyst nematode resistance in soybean. Crop Science, 44, 1121–1131. https://doi.org/10.2135/cropsci2004.1121
Concibido, V., La Vallee, B., McLaaid, P., Pineda, N., Meyer, J., Hummel, L., Yang, K., Wu, K., & Delannay, X. (2003). Introgroession of a quantitative trait locus for yield from Glycine soja into commercial soybean cultivars. Theoretical and Applied Genetics, 106, 575–582. https://doi.org/10.1007/s00122-002-1071-5
Cook, D. E., Bayless, A. M., Wang, K., Guo, X., Song, Q., Jiang, J., & Bent, A. F. (2014). Distinct copy number, coding sequence, and locus methylitation patterns underlie Rhg1-mediated soybean resistance to soybean cyst nematode. Plant Physiology, 165, 630–647. https://doi.org/10.1104/pp.114.235952
Cook, D. E., Lee, T. G., Guo, X., Melito, S., Wang, K., Bayless, K., Wang, J., Hughes, T. J., Willis, D. K., Clemente, T. E., Diers, B. W., Jiang, J. K., Hudson, M. E., & Bent, A. F. (2012). Copy number variation of multiple genes at Rhg1 mediates nematode resistance in soybean. Science, 338, 1206–1209. https://doi.org/10.1126.science.1228746
Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth, G. T., Sherry, S. T., McVean, G., Durbin, R., & 1000 Genomes Project Analysis Group. (2011). The variant call format and VCFtools. Bioinformatics, 27, 2156–2158. https://doi.org/10.1093/bioinformatics/btr330
Davis, E. L., Hussey, R. S., Mitchum, M. G., & Baum, T. J. (2008). Parasitism proteins in nematode–plant interactions. Current Opinion in Plant Biology, 11, 360–366. https://doi.org/10.1016/j.pbi.2008.04.003
Donald, P., Pierson, P., St Martin, S., Sellers, P., Noel, G., MacGuidwin, A., Faghihi, J., Ferris, R., Grau, R., Jardine, J., Melakeberhan, H., Niblack, T. L., Stienstra, W. C., Tylka, G. L., Wheeler, T. A., & Wyson, D. (2006). Accessing Heterodera glycines—Resistant and susceptible cultivar yield response. The Journal of Nematology, 38, 76–82.
Dong, J., Zielinski, R. E., & Hudson, M. E. (2020). t-SNAREs bind the Rhg1 α-SNAP and mediate soybean cyst nematode resistance. The Plant Journal, 104, 318–331. https://doi.org/10.1111/tpj.14923
Gardner, M., Dhiroso, A., Johnson, N., Davis, E. L., Baum, T. J., Korkin, D., & Mitchum, M. G. (2018). Novel global effector mining from the transcriptome of early life stages of the soybean cyst nematode Heterodera glycines. Scientific Reports, 8, 2505. https://doi.org/10.1038/s41598-018-20536-5
Gibson, D. G., Young, L., Chung, R. Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules in vitro as a digital mammalian cell. Nature Methods, 6, 343–345. https://doi.org/10.1038/nmeth.1318
Graham, P. H., & Vance, C. P. (2003). Legumes: Importance and constraints to greater use. The Journal of Nematology, 35, 1015–1029. https://doi.org/10.2135/jnematol2003.1015
Hesler, L. S. (2013). Resistance to soybean aphid among wild soybean lines under controlled conditions. Crop Protection, 53, 139–146. https://doi.org/10.1016/j.cropro.2013.06.016
Hoeft, C., & Hückelhoven, R. (2008). Enemy at the gates: Traffic at the plant cell pathogen interface. *Cellular Microbiology*, 10, 2400–2407. https://doi.org/10.1111/j.1462-5822.2008.01238.x

Huson, D. H., & Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, 23, 254–267. https://doi.org/10.1093/molbev/msj030

Hyten, D. L., Song, Q., Zhu, Y., Choi, I. Y., Nelson, R. L., Costa, J. M., Specht, J. E., Shoemaker, R. C., & Cregan, P. B. (2006). Impacts of genetic bottlenecks on soybean genome diversity. *Proceedings of the National Academy of Sciences*, 103, 16666–16671. https://doi.org/10.1073/pnas.0604379103

Inada, N., & Ueda, T. (2014). Membrane trafficking pathways and their roles in plant–microbe interactions. *Plant and Cell Physiology*, 55, 672–686. https://doi.org/10.1038/pccu046

Jahn, R., & Scheller, R. H. (2006). SNAREs—Engines for membrane fusion. *Nature Reviews Molecular Cell Biology*, 7, 631–643. https://doi.org/10.1038/nrm2002

Kabelka, E. A., Carlson, S. R., & Diers, B. W. (2005). Localization of two loci that confer resistance to soybean cyst nematode from *Glycine soja* PI 468916. *Crop Science*, 45, 2473–2481. https://doi.org/10.2135/cropsici2005.0027

Kabelka, E. A., Carlson, S. R., & Diers, B. W. (2006). *Glycine soja* PI 468916 SCN resistance loci’s associated effects on soybean seed yield and other agronomic traits. *Crop Science*, 46, 622–629. https://doi.org/10.2135/cropsici2005.06-0131

Keim, P., Olson, T. C., & Shoemaker, R. C. (1988). A rapid protocol for isolating soybean DNA. *Soybean Genetic Newsletter*, 15, 150–152.

Kim, M., & Diers, B. W. (2013). Fine mapping of the SCN resistance QTL and from PI 468916. *Crop Science*, 53, 775–785. https://doi.org/10.2135/cropsici2012.07.0425

Kim, M., Hyten, D. L., Bent, A. F., & Diers, B. W. (2010). Fine mapping of the SCN resistance locus *rhg1*-b from PI 88788. *The Plant Genome*, 3. https://doi.org/10.3835/plantgenome2010.02.0001

Koenning, S. R., & Wrather, J. A. (2010). Suppression of soybean yield potential in the Continental United States by plant diseases from 2006 to 2009. *Plant Health Progress*, 11, 5. https://doi.org/10.1094/PHP-2010-1122-01-01-RS

Kopisch-Obuch, F. J., & Diers, B. W. (2005). Segregation at the SCN resistance locus *rhg1* in soybean is distorted by an association between the resistance allele and reduced field emergence. *Theoretical and Applied Genetics*, 112, 199–207. https://doi.org/10.1007/s00122-005-0104-2

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549. https://doi.org/10.1093/molbev/msy096

Lakhssassi, N., Liu, S., Bekal, S., Zhou, Z., Colantonio, V., Lambert, K., Barakat, A., & Meksem, K. (2017). Characterization of the soluble NSF attachment protein gene family identifies two members involved in additive resistance to a plant pathogen. *Scientific Reports*, 7, 45226. https://doi.org/10.1038/srep45226

Lakhssassi, N., Piya, S., Bekal, S., Liu, S., Zhou, Z., Bergounioux, C., & Meksem, K. (2020). A pathogenesis-related protein GmPR08-Bet VI promotes a molecular interaction between the GmSHMT08 and GmSNAP18 in resistance to *Heterodera glycines*. *Plant Biotechnology Journal*, 18, 1810–1829. https://doi.org/10.1111/pbi.13343

Lee, J. D., Shannon, J. G., Vuong, T. D., & Nguyen, H. T. (2009). Inheritance of salt tolerance in wild soybean (*Glycine soja* Sieb. and Zucc.) accession PI483463. *Journal of Heredity*, 100, 798–801. https://doi.org/10.1093/jhered/esp027

Lee, J. S., Yoo, M., Jung, J. K., Bilyeu, K. D., Lee, J. D., & Kang, S. (2015a). Detection of novel QTLs for foxglove aphid resistance in soybean. *Theoretical and Applied Genetics*, 128, 1481–1488. https://doi.org/10.1007/s00122-015-2519-8

Lee, T. G., Diers, B. W., & Hudson, M. E. (2016). An efficient method for measuring copy number variation applied to improvement of nematode resistance in soybean. *The Plant Journal*, 88, 143–153. https://doi.org/10.1111/tpj.13240

Lee, T. G., Kumar, I., Diers, B. W., & Hudson, M. E. (2015b). Evolution and selection of *Rhg1*, a copy-number variant nematode-resistance locus. *Molecular Ecology*, 24, 1774–1791. https://doi.org/10.1111/mec.13138

Li, H., & Durbin, R. (2010). Fast and accurate read-length alignment with Burrows–Wheeler transform. *Bioinformatics*, 26, 589–595. https://doi.org/10.1093/bioinformatics/btp698

Liu, S., Kandoth, P. K., Lakhssassi, N., Kang, J., Colantonio, V., Heinz, R., Yeckel, G., Zhou, Z., Bekal, S., DaPrrich, J., Rotter, B., Cianzio, S., Mitchum, M. G., & Meksem, K. (2017). The soybean *GmSNAP18* gene underlies two types of resistance to soybean cyst nematode. *Nature Communications*, 8, 14822. https://doi.org/10.1038/ncomms14822

Liu, S., Kandoth, P. K., Warren, S. D., Yeckel, G., Heinz, R., Alden, J., Yang, C., Jamai, A., El-Mellouki, T., Juvale, P. S., Hill, J., Baum, T. J., Cianzio, S., Whitham, S. A., Korkin, D., Mitchum, M. G., & Meksem, K. (2012). A soybean cyst nematode resistance gene points to a new mechanism of plant resistance to pathogens. *Nature*, 492, 256–260. https://doi.org/10.1038/nature11651

Liu, Y., Du, H., Li, P., Shen, Y., Peng, H., Liu, S., Shi, M., Jiang, X., Li, Y., Wang, Z., Zhu, B., Han, B., Liang, C., & Tian, Z. (2020). Pan-genome of wild and cultivated soybeans. *Cell*, 182, 162–176. https://doi.org/10.1016/j.cell.2020.05.023

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal*, 17, 10–12. https://doi.org/10.14806/embnetj.17.1.200

Marz, K. E., Lauer, J. M., & Hanson, P. I. (2003). Defining the SNARE complex binding surface of α-SNAP. *Journal of Biological Chemistry*, 278, 27000–27008. https://doi.org/10.1074/jbc.M302003200

Matsye, P. D., Lawrence, G. W., Youssef, R. M., Kim, K. H., Lawrence, K. S., Matthews, B. F., & Klink, V. P. (2012). The expression of a naturally occurring, truncated allele of an α-SNAP gene suppresses plant parasitic nematode infection. *Plant Molecular Biology*, 80, 131–155. https://doi.org/10.1007/s11103-012-9932-z

McCarville, M. T., Marett, C. C., Mullaney, M. P., Gebhart, G. D., & Tytlka, G. L. (2017). Increase in soybean cyst nematode virulence and reproduction on resistant soybean varieties in Iowa From 2001 to 2015 and the effects on soybean yields. *Plant Health Progress*, 18, 146–155. https://doi.org/10.1094/PHP-RE-16-0062

Meksem, K., Pantazopoulos, P., Njiti, V. N., Hyten, L. D., Arelli, P. R., & Lightfoot, D. A. (2001). ‘Forrest’ resistance to the soybean cyst nematode resistance locus. *Theoretical and Applied Genetics*, 103, 798–801. https://doi.org/10.1007/s001220100597

Melito S., Heuberger A. L., Cook D., Diers B. W., MacGuidwin A. E., & Bent A. F. (2010). A nematode demographics assay in transgenic roots reveals no significant impacts of the Rhg locus LRR-Kinase on soybean cyst nematode resistance. *BMC Plant Biology*, 10(1), 104. https://doi.org/10.1186/1471-2229-10-104
Mitchum, M. G. (2016). Soybean resistance to the soybean cyst nematode *Heterodera glycines*: An update. *Phytopathology, 106*, 1444–1450. https://doi.org/10.1094/PHYTO-06-16-0227-RVW

Naydenov, N. G., Harris, G., Brown, B., Schaefer, K. L., Das, S. K., Fisher, P. B., & Ivanov, A. I. (2011). Loss of soluble N-ethylmaleimide-sensitive factor attachment protein α (αeSNAP) induces epithelial cell apoptosis via down-regulation of Bel-2 expression and disruption of the Golgi. *Journal of Biological Chemistry*, 287, 5928–5941. https://doi.org/10.1074/jbc.M111.278358

Niblack, T. L., Colgrove, A. L., Colgrove, K., & Bond, J. P. (2008). Shift in virulence of soybean cyst nematode is associated with use of resistance from PI 88788. *Plant Health Progress, 9*, 29. https://doi.org/10.1094/PHP-2008-0118-01-RS

Niblack, T. L., Lambert, K. N., & Tyka, G. L. (2006). A model plant pathogen from the kingdom Animalia: *Heterodera glycines*, the soybean cyst nematode. *Annual Review of Phytopathology, 44*, 283–303. https://doi.org/10.1146/annurev.phyto.44.040204.140218

Niblack, T. L., Tyka, G. L., Arelli, P., Bond, J., Diers, B., Donald, P., Faghhihi, J., Ferris, V. R., Gallo, K., Heinz, R. D., Lopez-Nicora, H., Von Qualen, R., Welacky, T., & Wilcox, J. (2009). A standard greenhouse method for assessing soybean cyst nematode resistance in soybean: SCE08 (standardized cyst evaluation 2008). *Plant Health Progress, 10*, 33. https://doi.org/10.1094/PHP-2009-0513-01-RV

Patil, G. B., Lakhssassi, N., Wan, J., Song, L., Zhou, Z., Klepadlo, M., Vuong, T. D., Stec, A. O., Kahl, S. S., Colantonia, V., Valliyodan, B., Rice, J. H., Piya, S., Hewei, T., Stupar, R. M., Meksem, S., & Nguyen, H. T. (2019). Whole-genome re-sequencing reveals the impact of the interaction of copy number variants of the *rhg1* and *Rhg4* genes on broad-based resistance to soybean cyst nematode. *Plant Biotechnology Journal, 17*, 1595–1611. https://doi.org/10.1111/pbi.13086

Poland, J. A., Brown, P. J., Sorrells, M. E., & Jannink, J. L. (2012). Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. *PLoS ONE, 7*, e32253. https://doi.org/10.1371/journal.pone.0032253

Poplin, R., Ruano-Rubio, V., DePristo, M. A., Fennell, T. J., Carneiro, M. O., Van der Auwera, G. A., Klig, D. E., Gauthier, L. D., Levy-Moonshine, A., Rozen, D., Shakir, K., Thibault, J., Chandran, S., Levy-Zugimann, A., & Niu, W. (2017). Scaling accurate genetic variant discovery to tens of thousands of samples. *BioRxiv*. https://doi.org/10.1101/201178

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D. L., Song, Q., Thelen, J. J., Cheng, J., Xu, D., Hellsten, U., May, G. D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M. K., Sandhu, D., Valliyodan, B., … Jackson, S. A. (2010). Genomic sequence of the paleopolyploid soybean. *Nature, 463*, 178–183. https://doi.org/10.1038/nature08670

Shi, Z., Liu, S., Noe, J., Arelli, P., Meksem, K., & Li, Z. (2015). SNP identification and marker assay development for high-throughput selection of soybean cyst nematode resistance. *BMC Genomics, 16*, 314. https://doi.org/10.1186/s12864-015-1531-3

Song, Q., Hyten, D. L., Jia, G., Quigley, C. V., Fickus, E. W., Nelson, R. L., & Cregan, P. B. (2013). Development and evaluation of SoySNP50K, a high-density genotyping array for soybean. *PLoS ONE, 8*, e54985. https://doi.org/10.1371/journal.pone.0054985

Song, Q., Hyten, D. L., Jia, G., Quigley, C. V., Fickus, E. W., Nelson, R. L., & Cregan, P. B. (2015). Fingerprinting soybean germplasm and its utility in genomic research. *G3: Genes, Genomes, Genetics, 5*, 1999–2006. https://doi.org/10.1534/g3.115.019000

Stacey, G. (Ed.). (2010). *Genetics and genomics of soybean*. Springer.

Stecher, G., Tamura, K., & Kumar, S. (2020). Molecular evolutionary genetics analysis (MEGA) for macOS. *Molecular Biology and Evolution, 37*, 1237–1239. https://doi.org/10.1093/molbev/msz312

Torkamaneh, D., Laroche, J., Valliyodan, B., O’Donoughue, L., Cober, E., Rajcan, I., Abdelnoor, R. V., Sreedasyam, A., Schmutz, J., Nguyen, H. T., … Belzile, F. (2019). Soybean haplotype map (GmHapMap): A universal resource for soybean translational and functional genomics. *bioRxiv* 534578. https://doi.org/10.1101/534578

Tran, D. T., Steketee, C. J., Boehm, J. D., Noe, J., & Li, Z. (2019). Genome-wide association analysis pinpoints additional major genomic regions conferring resistance to soybean cyst nematode (*Heterodera glycines* Ichinohe). *Frontiers in Plant Science, 10*, 401. https://doi.org/10.3389/fpls.2019.00401

Tuberosa, R. (2012). Phenotyping for drought tolerance of crops in the genomics era. *Frontiers in Physiology, 3*, 347. https://doi.org/10.3389/fphys.2012.00347

Usovssky, M., Ye, H., Vuong, T. D., Patil, G. B., Wan, J., Zhou, L., & Nguyen, H. T. (2020). Fine-mapping and characterization of *qSCN18*, a novel QTL controlling soybean cyst nematode resistance in PI 567516C. *Theoretical and Applied Genetics, 134*, 621–631. https://doi.org/10.1007/s00122-020-03718-6

Varley, K. E., & Mitra, R. D. (2010). Bisulfite patch PCR enables multiplexed sequencing of promoter methylation across cancer samples. *Genome Research, 20*, 1279–1287. https://doi.org/10.1101/gr.101212.109

Vuong, T. D., Sonah, H., Meinhardt, C. G., Deshmukh, R., Kadam, S., Nelson, R. L., et al., & Nguyen, H. T. (2015). Genetic architecture of cyst nematode resistance revealed by genome-wide association study in soybean. *BMC Genomics, 16*, 593. https://doi.org/10.1186/s12864-015-1811-y

Wang, D., Diers, B. W., Arelli, P. R., & Shoemaker, R. C. (2001). Loci controlling soybean cyst nematode race-3 resistance loci in the soybean PI 437.654. *Theoretical and Applied Genetics, 91*, 574–581. https://doi.org/10.1007/s00122-001-02382-8

Winter, S. M. J., Shelp, B. J., Anderson, T. R., Welacky, T. W., & Rajcan, I. (2007). QTL associated with horizontal resistance to soybean cyst nematode in *Glycine soja* PI464925B. *Theoretical and Applied Genetics, 114*, 461–472. https://doi.org/10.1007/s00122-006-0446-4

Wu, X. Y., Zhou, G. C., Chen, Y. X., Wu, P., Liu, L. W., Ma, F. F., Wu, M., Liu, C. C., Zeng, Y. J., Chu, A. E., Hang, Y. Y., Chen, J. Q., & Wang, B. (2016). Soybean cyst nematode resistance emerged via artificial selection of duplicated serine hydroxymethyltransferase genes. *Frontiers in Plant Science, 7*, 998. https://doi.org/10.3389/fpls.2016.00998

Xie, M., Chung, C. Y. L., Li, M. W., Wong, F. L., Wang, X., Liu, A., Wang, Z., Yang, L., Deng, T., He, L., Chen, L., Fu, A., Ding, Q., He, J., Chung, G., Isobe, S., Tanabata, T., Valliyodan, B., Nguyen, T., & Lam, H. M. (2019). A reference-grade wild soybean genome. *Nature Communications, 10*, 1216. https://doi.org/10.1038/s41467-019-09142-9
Yu, N., & Diers, B. W. (2017). Fine mapping of the SCN resistance QTL *cqSCN-006* and *cqSCN-007* from *Glycine soja* PI 468916. *Euphytica*, 213, 54. https://doi.org/10.1007/s10681-016-1791-2

Yu, N., Lee, T. G., Rosa, D. P., Hudson, M., & Diers, B. W. (2016). Impact of *Rhg1* copy number, type, and interaction with *Rhg4* on resistance to *Heterodera glycines* in soybean. *Theoretical and Applied Genetics*, 129, 2403–2412. https://doi.org/10.1007/s00122-016-2779-y

Zhang, H., Kjemtrup-Lovelace, S., Li, C., Luo, Y., Chen, L. P., & Song, B. H. (2017a). Comparative RNA-Seq analysis uncovers a complex regulatory network for soybean cyst nematode resistance in wild soybean (*Glycine soja*). *Scientific Reports*, 7, 9699. https://doi.org/10.1038/s41598-017-09945-0

Zhang, H., Li, C., Davis, E. L., Wang, J., Griffin, J. D., Kofsky, J., & Song, B. H. (2016). Genome-wide association study of resistance to soybean cyst nematode (*Heterodera glycines*) HG Type 2.5.7 in wild soybean (*Glycine soja*). *Frontiers in Plant Science*, 7, 1214. https://doi.org/10.3389/fpls.2016.01214

Zhang, H., Song, Q., Griffin, J. D., & Song, B. H. (2017b). Genetic architecture of wild soybean (*Glycine soja*) response to soybean cyst nematode (*Heterodera glycines*). *Molecular Genetics and Genomics*, 292, 1257–1265. https://doi.org/10.1007/s00438-017-1345-x

Zhao, C., Slevin, J. T., & Whiteheart, S. W. (2007). Cellular functions of NSF: Not just SNAPs and SNAREs. *FEBS Letters*, 581, 2140–2149. https://doi.org/10.1016/j.febslet.2007.03.032

Zhao, M., Wu, S., Zhou, Q., Vivona, S., Cipriano, D. J., Cheng, Y., & Brunger, A. T. (2015). Mechanistic insights into the recycling machine of the SNARE complex. *Nature*, 518, 61–67. https://doi.org/10.1038/nature14148

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

---

**How to cite this article:** Grunwald DJ, Zapotocny RW, Ozer S, Diers BW, Bent AF. Detection of rare nematode resistance *Rhg1* haplotypes in *Glycine soja* and a novel *Rhg1* α-SNAP. *Plant Genome*. 2022;15:e20152. https://doi.org/10.1002/tpg2.20152