



An atypical N-ethylmaleimide sensitive factor enables the viability of nematode-resistant *Rhg1* soybeans

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N-ethylmaleimide sensitive factor (NSF) and α -soluble NSF attachment protein (α -SNAP) are essential eukaryotic housekeeping proteins that cooperatively function to sustain vesicular trafficking. The “resistance to *Heterodera glycines* 1” (*Rhg1*) locus of soybean (*Glycine max*) confers resistance to soybean cyst nematode, a highly damaging soybean pest. *Rhg1* loci encode repeat copies of atypical α -SNAP proteins that are defective in promoting NSF function and are cytotoxic in certain contexts. Here, we discovered an unusual NSF allele (*Rhg1*-associated NSF on chromosome 07; *NSF_{RAN07}*) in *Rhg1*⁺ germplasm. *NSF_{RAN07}* protein modeling to mammalian NSF/ α -SNAP complex structures indicated that at least three of the five *NSF_{RAN07}* polymorphisms reside adjacent to the α -SNAP binding interface. *NSF_{RAN07}* exhibited stronger *in vitro* binding with *Rhg1* resistance-type α -SNAPs. *NSF_{RAN07}* coexpression *in planta* was more protective against *Rhg1* α -SNAP cytotoxicity, relative to WT *NSF_{CH07}*. Investigation of a previously reported segregation distortion between chromosome 18 *Rhg1* and a chromosome 07 interval now known to contain the *Glyma.07G195900* NSF gene revealed 100% coinheritance of the *NSF_{RAN07}* allele with disease resistance *Rhg1* alleles, across 855 soybean accessions and in all examined *Rhg1*⁺ progeny from biparental crosses. Additionally, we show that some *Rhg1*-mediated resistance is associated with depletion of WT α -SNAP abundance via selective loss of WT α -SNAP loci. Hence atypical coevolution of the soybean SNARE-recycling machinery has balanced the acquisition of an otherwise disruptive housekeeping protein, enabling a valuable disease resistance trait. Our findings further indicate that successful engineering of *Rhg1*-related resistance in plants will require a compatible NSF partner for the resistance-conferring α -SNAP.

plant disease resistance | α -SNAP | NSF | soybean cyst nematode | *Rhg1*

Cyst nematodes infest the roots of many valuable crops and establish elaborate feeding structures (1). Soybean cyst nematode (*Heterodera glycines*; SCN) is a highly damaging soybean pest and causes annual US yield losses of over \$1 billion US dollars (2–5). SCN parasitizes host roots by secreting a complex arsenal of effector molecules that reprogram host root cells and trigger fusion with adjacent host cells, forming a large unicellular feeding site termed a syncytium (6–8). The soybean “resistance to *Heterodera glycines* 1” (*Rhg1*) locus is very widely used by soybean growers to restrict SCN feeding site formation, thereby reducing yield loss (4, 9). The genes at *Rhg1* do not encode proteins normally associated with disease resistance (4, 10–12). Instead, resistance is mediated by copy number variation of multiple genes at the *Rhg1* locus, one of which encodes an α -soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (α -SNAP) with unusual C-terminal polymorphisms (10, 11, 13).

α -SNAP (Sec17 in yeast) is a functionally conserved eukaryotic housekeeping protein that works in concert with NSF (Sec18 in yeast). α -SNAP and NSF promote cellular vesicular trafficking by mediating the disassembly and reuse of soluble NSF attachment protein receptor (SNARE) protein complexes that form when t-SNARE and v-SNARE proteins associate during vesicle docking and fusion (14–16). We recently discovered that the soybean resistance-associated α -SNAPs encoded by *Rhg1* are unusual α -SNAP proteins that bind less well to wild-type (WT)

NSF and, when expressed in *Nicotiana benthamiana*, disrupt vesicle trafficking and eventually cause cell death (17). The relative abundance of *Rhg1*-encoded defective α -SNAP variants increases substantially within developing host syncytial cells, apparently disrupting syncytium viability and thereby restricting nematode growth and reproduction (17). SCN-resistant soybeans carry WT α -SNAP genes at other loci that can functionally complement the *Rhg1* resistance-type α -SNAPs in a dosage-dependent manner (17). However, the capacity of soybean varieties to yield well despite expression of cytotoxic *Rhg1* resistance-type α -SNAPs throughout the plant is not fully explained.

The complex *Rhg1* locus on soybean chromosome 18 is a tandemly repeated block of four genes: *Glyma.18G022400*, *Glyma.18G022500*, *Glyma.18G022600*, and *Glyma.18G022700*. SCN-susceptible soybeans carry only a single copy of the above four genes, including a *Glyma.18G022500* α -SNAP gene whose product matches the WT α -SNAP consensus and maintains normal NSF interactions (10, 13, 17). Resistance-conferring *Rhg1* loci group into two structural classes based on the type of α -SNAP polymorphisms they encode, which also correlates with the copy number of *Rhg1* repeats that are present (11, 13) (*SI Appendix*, Table S1). *Rhg1_{HC}* (high-copy) loci carry four or more and frequently 9 or 10 *Rhg1* repeats, and *Rhg1_{LC}* (low-copy) loci carry three or fewer *Rhg1* repeats. *Rhg1_{HC}* (also known as *rhg1-b*)

Significance

N-ethylmaleimide sensitive factor (NSF) and α -soluble NSF attachment protein (α -SNAP) are key components of vesicle trafficking systems and are conserved across eukaryotes. This study shows that these two essential housekeeping proteins have coevolved toward atypical forms in soybean to confer resistance to a highly damaging nematode pathogen while balancing plant fitness. We report discovery of a naturally occurring NSF variant carrying unusual polymorphisms that enhance interaction with and assuage the cytotoxicity of the *Rhg1* resistance-associated α -SNAPs. Pathogen selection pressure has apparently driven this rewiring of multiple components of the conserved SNARE recycling machinery. Useful introduction of the agriculturally valuable *Rhg1* resistance source into other plants is likely to require a cofunctional NSF protein partner.

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Conflict of interest statement: A patent application covering the presently described work has been filed by the Wisconsin Alumni Research Foundation.

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and *Rhg1_{LC}* (also known as *rhg1-a*) encode distinct α -SNAP variants that are impaired in normal α -SNAP–NSF interactions (17) (Fig. 1A). All *Rhg1_{HC}* loci examined to date also carry a single *Rhg1* repeat that encodes a WT α -SNAP adjacent to multiple repeats that encode resistance-type α -SNAPs, while *Rhg1_{LC}* loci encode only resistance-type α -SNAPs and no WT α -SNAP (10, 11, 13) (Fig. 1A). Plants carrying *Rhg1_{HC}* or *Rhg1_{LC}* loci exhibit elevated transcript abundance for the repeat genes that correlates approximately with copy number, including for the *Rhg1* α -SNAP gene (10, 13). Collectively, the above findings suggest that modulation of vesicle trafficking and cell health at the SCN feeding site is at least one core mechanism of *Rhg1*-mediated SCN resistance.

Two other genes within the *Rhg1* repeat were reported by Cook et al. (10) to contribute to *Rhg1_{HC}*-mediated SCN resistance. *Glyma.18G022400* encodes an amino acid permease-like protein and *Glyma.18G022700* encodes a wound-inducible protein otherwise lacking annotated domains or predicted functions; their molecular function in SCN resistance remains unknown. Liu et al. (18) recently provided evidence that the *Rhg1_{LC}* α -SNAP may function differently than the *Rhg1_{HC}* α -SNAP.

The eukaryotic endomembrane network is an intricate sorting and secretion system that ferries cargoes between cellular compartments using transport vesicles. Cognate SNARE proteins on

the surface of vesicle and target membranes drive membrane fusion by “zippering” into stable bundles (SNARE complexes), which pull the membranes together (14, 19). The role of α -SNAP and NSF as dedicated SNARE-recycling chaperones has been studied extensively (14, 19–22). NSF is an “ATPases associated with various cellular activities” (AAA⁺) family protein with three domains: the N domain that binds and interacts with the C terminus of the α -SNAP cochaperone, the D1 ATPase domain that couples ATP hydrolysis to SNARE complex remodeling, and the D2 ATPase domain that mediates NSF hexamerization (23–25). The α -SNAP proteins are required by NSF to cochaperone SNARE remodeling. The α -SNAP serves both as an adaptor for NSF binding to SNARE complexes and as a stimulator of the NSF D1 domain ATPase activity that powers SNARE remodeling/recycling (15). Beyond disassembling SNARE complexes, additional roles of α -SNAP and NSF have been reported, including binding to trans-SNARE complexes to accelerate fusion (26), as well as binding of channels and other receptors and regulation of apoptosis (20, 27–30). The structure and function of α -SNAP, NSF, and SNARE proteins has been elucidated in substantial detail, including cryo-EM structures for 20S complexes that consist of a four-helix SNARE bundle, four α -SNAPs, and six NSFs in various conformational states (15, 21).

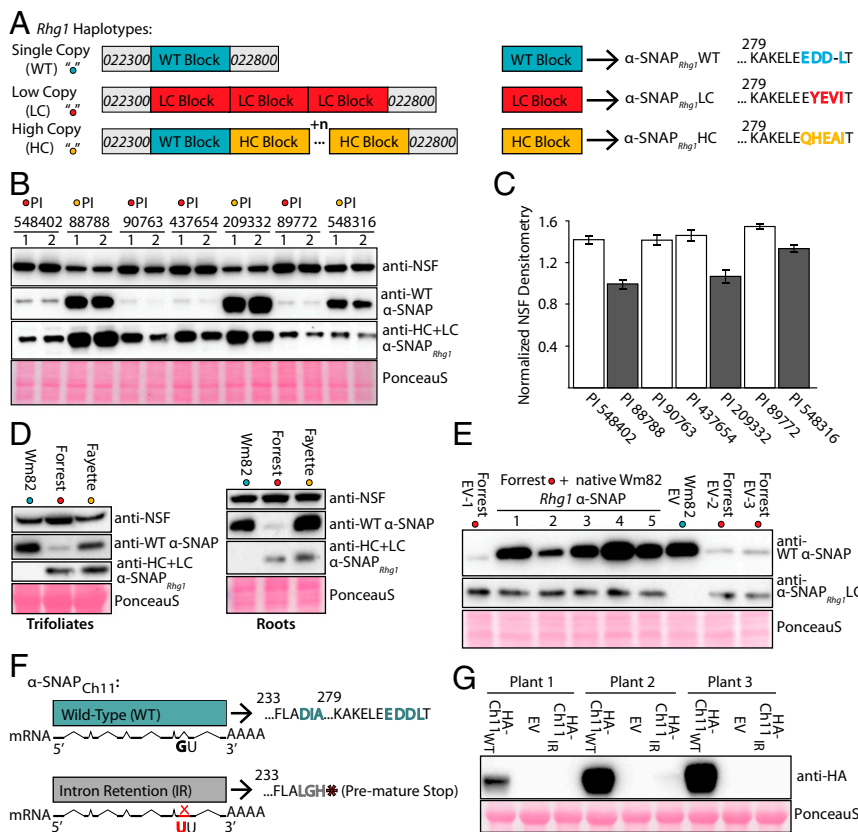


Fig. 1. WT α -SNAP proteins are much less abundant while NSF is more abundant in *Rhg1_{LowCopy}* soybeans. (A) Schematic of *Rhg1* haplotype classes. (Left) *Rhg1* WT (shown blue), *Rhg1* LC (shown red), *Rhg1* HC (shown orange; *n* = variable HC-type repeat numbers); not drawn to scale. The C-terminal amino acid polymorphisms encoded by the *Rhg1* α -SNAPs are shown at Right. HC *Rhg1* haplotypes retain a single WT-like *Rhg1* repeat. (B) Immunoblot of WT α -SNAPs, *Rhg1_{LC}* resistance-type α -SNAPs and NSF in roots of soybean HG test varieties (two samples for each genotype). *Rhg1_{LC}* varieties (Peking), PI 548402 (Peking), PI 89772, PI 437654, PI 90763; *Rhg1_{HC}* varieties (orange dot): PI 88788 (9 copies), PI 209332 (10 copies), PI 548316 (7 copies). PonceauS staining shows similar loading of total protein. (C) Densitometry indicating total NSF expression in HG type test lines. (D) Like B, but immunoblots for trifoliolate leaves or roots of Wm82 and modern *Rhg1_{LC}* and *Rhg1_{HC}* varieties Forrester and Fayette. (E) Immunoblots for total WT α -SNAPs and α -SNAP_{*Rhg1_{LC}*} in Forrester (*Rhg1_{LC}*) transgenic roots transformed with an empty vector (EV; three transgenic lines) or with the native Wm82 α -SNAP_{*Rhg1*}WT locus (five transgenic lines), or in WT Wm82 roots transformed with EV. (F) Schematic of chromosome 11 α -SNAP alleles with exon/intron models, and nucleotide and amino acid polymorphisms. (G) The encoded α -SNAP_{Ch11} intron retention protein, unlike the WT α -SNAP_{Ch11}, does not accumulate. Anti-HA immunoblot of total protein from *N. benthamiana* leaves is agroinfiltrated to express empty vector, N-HA- α -SNAP_{Ch11}, or N-HA- α -SNAP_{Ch11}-IR (intron retention). PonceauS staining shows similar loading of total protein.

Although most animal genomes carry a single NSF and a single α -SNAP gene, polyploidization and other events have caused most plant genomes to encode multiple NSF and α -SNAP genes (31). The reference Williams 82 (Wm82) soybean genome (32) encodes seven SNAP family members: five putative α -SNAPs and two putative γ -SNAPs. Soybean also encodes two unlinked NSF genes, *Glyma.07G195900* and *Glyma.13G180100*. As in animals, plants contain >100 genes encoding diverse SNARE and SNARE-like proteins (14, 33). Unlike plant SNARE proteins [including SNAREs with potentially confusing names such as synaptosomal-associated protein 25 (SNAP-25) and soluble N-ethylmaleimide-sensitive factor adaptor protein 33 (SNAP33)], there are very few published studies of plant NSF, α -SNAP, or γ -SNAP proteins (10, 13, 17, 18, 34–37). However, close analysis of recombinant-inbred lines has recently shown that a gene at or linked to the soybean chromosome 11 locus encoding an α -SNAP makes a minor contribution to SCN resistance in the Peking (*Rhg1_{LC}* + *Rhg4*) genetic background (38). Other previous work (37) had identified an allele encoding a splice-variant α -SNAP in this genetic background, although that work misidentified it as an allele of the chromosome 18 *Rhg1* locus despite it now being known to be a chromosome 11 α -SNAP allele (13, 38).

In the present study, we demonstrate that evolution/selection of both *Rhg1_{LC}* and the chromosome 11 α -SNAP gene *Glyma.11G234500* has had major impacts on the relative abundance of WT α -SNAP proteins in the *Rhg1_{LC}* genetic background. We also examined soybean NSF proteins. We discovered an unusual NSF protein in *Rhg1*-containing lines that is unlike that encoded in the soybean Wm82 reference genome or any publicly available plant reference genomes. We found that this variant NSF_{RAN07} (*Rhg1*-associated NSF on chromosome 07; NSF_{RAN07}) protein contains unique N-domain polymorphisms that mitigate the cytotoxicity and poor NSF binding activity of the SCN resistance-conferring *Rhg1* α -SNAPs. We then noted that the genetic region containing this NSF and neighboring genes has been identified in previous SCN resistance mapping studies, including a 1995 study by Webb showing strong cosegregation with resistance-conferring *Rhg1* alleles (39, 40). More recently, a high-resolution 80-kb candidate gene interval was identified (41) but this segregation distortion at the chromosome 07 locus had remained unexplained. We therefore investigated soybean germplasm genotype data and recombinant inbred lines from *Rhg1⁺* × *rhg1⁻* parental crosses. We discovered strict coinheritance of NSF_{RAN07} alleles in plants homozygous for resistance-associated *Rhg1* haplotypes, demonstrating the functional necessity of NSF_{RAN07} for viable occurrence of SCN resistance-conferring *Rhg1*.

Results

WT α -SNAP Proteins Are Much Less Abundant While NSF Is More Abundant in *Rhg1_{LC}* Soybeans. We previously reported that the PI 88788-type high-copy (HC) *Rhg1* (*Rhg1_{HC}*) locus in soybean line “Fayette” drives a localized increase of resistance-type α -SNAP_{*Rhg1_{HC}*} protein to disrupt the developing SCN-induced syncytium (17). We also observed that endogenous NSF levels increased when resistance-associated *Rhg1* α -SNAP proteins were overexpressed in *N. benthamiana* (17). However, for lines carrying LC-type *Rhg1* (*Rhg1_{LC}*, “Peking-type”), the cellular balance of WT α -SNAP to α -SNAP_{*Rhg1_{LC}*} or NSF proteins was unknown. To investigate the relative abundances of WT and resistance-associated α -SNAPs, we used previously described anti- α -SNAP antibodies and performed immunoblots on the *Rhg1_{HC}* and *Rhg1_{LC}* soybean varieties commonly used to phenotype SCN resistance (the HG Type Test varieties; see *SI Appendix, Table S1*) (17, 42). We also examined the abundance of the α -SNAP cochaperone NSF in these samples, using an antibody raised to a conserved NSF region (17). Fig. 1A presents a schematic of the various *Rhg1* haplotypes as well as the C-terminal polymorphisms of *Rhg1* α -SNAPs encoded by the *Rhg1* repeat types. As shown in

Fig. 1B, immunoblots from root tissue indicated that WT α -SNAP protein levels in all tested *Rhg1_{LC}* lines (PI 548402/Peking, PI 90763, PI 437654, PI 89772) are dramatically reduced compared with the *Rhg1_{HC}* lines (PI 88788, PI 209332, PI 548316). As mentioned in the Introduction, the Wm82 soybean genome encodes five putative α -SNAPs, and the anti-WT- α -SNAP antibody was raised against the conserved C terminus shared by all of those predicted WT α -SNAP gene products but not the resistance-associated *Rhg1* α -SNAPs (17). In addition, one *Rhg1* repeat in *Rhg1_{HC}* haplotypes encodes a WT *Glyma.18G022500* α -SNAP protein and all other repeats encode a resistance-type *Rhg1* α -SNAP protein, while the *Rhg1_{LC}* repeats encode only resistance-type α -SNAP_{*Rhg1_{LC}*} proteins (Fig. 1A) (11, 13). The results of Fig. 1B did not match initial predictions; the tested *Rhg1_{LC}* soybean lines exhibit very low WT α -SNAP protein levels despite the presence of multiple α -SNAP genes at other loci.

We further discovered that total NSF protein abundance in the *Rhg1_{LC}* lines is increased compared with the *Rhg1_{HC}* lines PI 88788 and PI 209332 (Fig. 1B and *SI Appendix, Fig. S1A*). These differences in NSF abundance, across two independent experiments, were quantified using densitometry (Fig. 1C).

We then explored whether WT α -SNAP protein abundance is similarly reduced in a more recent agriculturally utilized *Rhg1_{LC}* soybean variety, “Forrest.” Immunoblots on both total leaf and root proteins from Wm82 (*Rhg1* single copy), Forrest (*Rhg1_{LC}*), and Fayette (*Rhg1_{HC}*) again revealed sharp decreases in total WT α -SNAP abundance in the *Rhg1_{LC}* source (Fig. 1D). Altogether, diminished WT α -SNAP protein levels were observed to be a shared trait of *Rhg1_{LC}* but not *Rhg1_{HC}* soybean varieties. In at least two previously studied *Rhg1_{LC}* varieties, as well as PI 548316, the chromosome 11 α -SNAP allele (*Glyma.11G234500*) carries a SNP at an intronic splice donor site (Fig. 1F and *SI Appendix, Table S1*), leading to intron retention and early translational termination, presumably truncating the protein (13, 37, 38). Hence a likely hypothesis for this strikingly low abundance is the absence of a WT- α -SNAP-encoding allele at *Rhg1_{LC}*, low or no product from the α -SNAP_{Ch11} allele whose transcript retains a translation-terminating intron, and a relatively minor contribution of protein from the other three putative α -SNAP-encoding loci.

Contributions to WT α -SNAP abundance were investigated further. First, we examined overall WT α -SNAP protein abundance when a locus encoding α -SNAP_{*Rhg1*}WT is ectopically placed into *Rhg1_{LC}* soybean lines. We cloned from Wm82 the genomic chromosome 18 (Ch18) *Glyma.18G022500* α -SNAP_{*Rhg1*}WT locus with its native promoter and terminator sequences, generated transgenic Forrest (*Rhg1_{LC}*) roots carrying this native α -SNAP_{*Rhg1*}WT locus, and assessed total WT α -SNAP protein levels using immunoblots (Fig. 1E). Transgenic addition of the Wm82 α -SNAP_{*Rhg1*}WT locus increased total WT α -SNAP protein expression in Forrest to levels similar to Wm82 empty vector controls (Fig. 1E). This result indicates that, if an appropriate gene is present, normal WT α -SNAP protein levels can develop in the *Rhg1_{LC}* genetic background.

Next, we examined α -SNAP protein production from the chromosome 11 (Ch11) WT locus from Wm82 vs. the Ch11 intron retention allele (α -SNAP_{Ch11-IR}) that is present in many soybean lines that carry *Rhg1_{LC}* on Ch18. The transcript from the intron retention allele encodes a premature stop codon (13, 37, 38) (Fig. 1F), but the abundance/stability of this putative α -SNAP protein was not known. As such, we cloned ORFs of both the WT α -SNAP_{Ch11} and the intron retention (α -SNAP_{Ch11-IR}) alleles, added an N-terminal HA tag, and examined transient protein expression in *N. benthamiana*. We observed that the HA- α -SNAP_{Ch11} WT protein, but not the truncated HA- α -SNAP_{Ch11-IR} protein, was readily detectable (Fig. 1G). The apparent instability of this truncated Ch11 α -SNAP was consistent with a homology model of WT α -SNAP_{Ch11} we generated using the yeast α -SNAP (Sec17) crystal structure (43), which predicted that the α -SNAP_{Ch11-IR} protein would terminate several residues into alpha-helix 12

(*SI Appendix, Fig. S1B*). We also noted a ~300-bp deletion occurring within the promoter of this allele. The presence and absence of this promoter deletion was verified using PCR on genomic DNA from Forrest (α -SNAP_{Ch11-IR}) and Wm82 (WT α -SNAP_{Ch11}), respectively (*SI Appendix, Fig. S1D*). Finally, as for the Ch18 locus tested in Fig. 1E, we cloned the Ch11 genomic WT locus of *Glyma.11G234500* (α -SNAP_{Ch11}) from Wm82 with native promoter and terminator and noted that presence of this native locus in transgenic roots of Forrest elevated total WT α -SNAP protein expression compared with empty vector controls (*SI Appendix, Fig. S1C*). Together, the findings of Fig. 1 and *SI Appendix, Fig. S1*, implicate the Ch18 and Ch11 WT α -SNAP loci as the major sources of total WT α -SNAP proteins in soybean and indicate that their combined absence from the examined *Rhg1_{LC}* varieties is responsible for the low levels of WT α -SNAP observed in Fig. 1B and D. The low abundance of WT α -SNAPs in lines carrying *Rhg1_{LC}* may improve SCN resistance but may also incur costs with respect to plant health and yield if other compensatory mechanisms for tolerance of *Rhg1_{LC}* are not also present.

A Unique NSF_{Ch07} Allele (NSF_{RAN07}) Is Present in Commonly Used *Rhg1*-Containing Accessions. NSF and α -SNAP are essential eukaryotic housekeeping proteins, and null mutations in either partner are lethal in animals, which typically encode only single copies of NSF or α -SNAP (31, 44–46). Because *Rhg1* resistance-type α -SNAPs (α -SNAP_{*Rhg1*LC} or α -SNAP_{*Rhg1*HC}) exhibit compromised binding to WT NSFs and are toxic at high doses in *N. benthamiana* (17), it was unclear how *Rhg1_{LC}* lines are viable given the diminished WT α -SNAP levels observed in Fig. 1. Since soybean is an ancestrally polyploid organism encoding multiple α -SNAP and NSF loci, we searched for alterations in the other α -SNAP or NSF loci by examining our previously generated whole-genome sequence (WGS) data from multiple *Rhg1*-containing varieties (13). For all five putative α -SNAP loci from *Rhg1_{LC}* varieties, we detected no obvious polymorphisms other than the previously mentioned *Glyma.11G234500* intron retention allele (*SI Appendix, Tables S1 and S2*) (13, 38).

Interestingly, a novel NSF allele was present at *Glyma.07G195900* (NSF_{Ch07}) among all six of the *Rhg1_{LC}* and *Rhg1_{HC}* lines examined, encoding five N-domain amino acid polymorphisms (R₄Q, N₂₁Y, S₂₅N, \wedge ₁₁₆F, and M₁₈₁I; \wedge = insertion) (Fig. 2A and *SI Appendix, Fig. S2A and Table S1*). Using cDNA from Forrest (*Rhg1_{LC}*), we cloned and sequenced this unique NSF_{Ch07} transcript and confirmed the five N-domain polymorphisms. Additionally, we designed two different PCR primer pairs at the encoded NSF polymorphisms and verified the presence of this unique NSF_{Ch07} allele, and the absence of the WT NSF_{Ch07} allele, in all *Rhg1* test lines (*SI Appendix, Fig. S2B*). Furthermore, using WGS data from the Soybean Nested Association Mapping (SoyNAM) project (47), we determined that this unique NSF_{Ch07} allele was also present in every *Rhg1*-containing NAM parent, while SCN-susceptible NAM parents carried the WT NSF_{Ch07} allele (*SI Appendix, Table S2*). We therefore named the protein from this *Rhg1*-associated allele of *Glyma.07G195900* “NSF_{RAN07}” for “*Rhg1*-associated NSF on chromosome 07.”

In addition to NSF_{RAN07}, an allele of the chromosome 13 *Glyma.13G180100* gene encoding an NSF_{Ch13} V₅₅₅I protein was found in some varieties, including SCN-susceptible soybeans, but it was not present in every *Rhg1_{LC}* or *Rhg1_{HC}* line (*SI Appendix, Table S2*). Normalized RNA sequencing reads from Wm82 indicate that both *Glyma.07G195900* and *Glyma.13G180100* are expressed similarly across examined plant tissues (*SI Appendix, Fig. S2C*) (48). *SI Appendix, Fig. S2A* provides the complete NSF_{RAN07} amino acid alignment to NSF_{Ch07} from the Wm82 genome.

The NSF_{RAN07} and *Rhg1* α -SNAP Polymorphisms Lie at the NSF/ α -SNAP Binding Interface. The NSF/ α -SNAP interface consists of complementary electrostatic patches located at the NSF N domain and α -SNAP C terminus (15, 21). The *Rhg1* polymorphisms of

both α -SNAP_{*Rhg1*HC} and α -SNAP_{*Rhg1*LC} are located at conserved C-terminal residues shown in other α -SNAPs to bind and stimulate NSF (13, 17, 49). These binding patches are conserved in yeast, animals, and plants, and interkingdom interactions between α -SNAP and NSF have been reported between mammals and yeast and plants, including soybean WT α -SNAP and Chinese hamster NSF (NSF_{CHO}) (17, 35, 36, 50). We performed homology modeling of NSF_{RAN07} to the NSF_{CHO} cryo-EM structure (21) [Protein Data Bank (PDB) ID code 3j97.1] that placed three of the NSF_{RAN07} polymorphisms, N₂₁Y, S₂₅N, and the \wedge ₁₁₆F insertion, adjacent to the NSF_{CHO} α -SNAP-binding residues R₁₀ and RK_{104–105} (Fig. 2B and *SI Appendix, Fig. S3A*). NSF_{RAN07} polymorphism R₄Q was outside of the model, and the final NSF_{RAN07} polymorphism M₁₈₁I was not located near the α -SNAP binding patches. Further homology modeling was conducted using the mammalian 20S cryo-EM structure (PDB ID code 3j97). In Fig. 2C and *SI Appendix, Fig. S4 A and B*, the complementary NSF and α -SNAP binding residues, and the NSF_{RAN07} and *Rhg1* α -SNAP polymorphisms, are colored. These results suggest that, upon α -SNAP binding, NSF_{RAN07} N₂₁Y, S₂₅N, and \wedge ₁₁₆F are close to the WT α -SNAP amino acid residues that are polymorphic in α -SNAP_{*Rhg1*HC} and α -SNAP_{*Rhg1*LC}. In separate bioinformatics work, we examined the NSF N-domain consensus in plants and determined that residues corresponding to N₂₁ and F₁₁₅ of WT soybean NSF are present in a majority of plant species, while neither the N₂₁Y nor the \wedge ₁₁₆F insertion of NSF_{RAN07} were detected in any available plant reference genome sequences (*SI Appendix, Fig. S3B*). Altogether, this modeling suggested that NSF_{RAN07} carries rare alterations at the α -SNAP binding interface that potentially influence interactions with the unusual C termini of *Rhg1* resistance-type α -SNAPs.

NSF_{RAN07} Polymorphisms Enhance Binding with *Rhg1* Resistance-Type α -SNAPs. In light of the above results, NSF_{RAN07} binding with *Rhg1* resistance-type α -SNAPs and α -SNAP_{*Rhg1*WT} was investigated. As in refs. 17 and 51, we produced recombinant NSF_{RAN07}, NSF_{Ch07}, and *Rhg1* α -SNAP proteins and performed in vitro binding assays. NSF_{RAN07} and NSF_{Ch07} binding was quantified using densitometry across three independent experiments (Fig. 2E). As previously reported (17), diminished NSF_{Ch07} binding was observed for α -SNAP_{*Rhg1*HC} and α -SNAP_{*Rhg1*LC}, compared with α -SNAP_{*Rhg1*WT} (Fig. 2D). The α -SNAP_{*Rhg1*HC} or α -SNAP_{*Rhg1*LC} binding of NSF_{RAN07}, on the other hand, was more similar to α -SNAP_{*Rhg1*WT} binding of NSF_{RAN07} and was increased ~30% relative to the binding of NSF_{Ch07} (Fig. 2D and E).

To investigate the contribution of the α -SNAP C terminus to NSF_{RAN07} binding, we tested NSF_{RAN07} binding to an otherwise WT α -SNAP that lacked the final 10 C-terminal residues (α -SNAP_{*Rhg1*WT_{1–279}}). Similar to the “no α -SNAP” binding controls, essentially no binding of either NSF_{Ch07} or NSF_{RAN07} with α -SNAP_{*Rhg1*WT_{1–279}} was observed (*SI Appendix, Fig. S4C*). To more specifically investigate the NSF binding contribution of just the C-terminal residues polymorphic in α -SNAP_{*Rhg1*LC} (Fig. 1A), we mutagenized α -SNAP_{*Rhg1*LC} from 286_{YEVI}289 to 286_{AAAA}289. Binding of either NSF_{Ch07} or NSF_{RAN07} to α -SNAP_{*Rhg1*LC} 286_{AAAA}289 was similar to “no α -SNAP” controls (*SI Appendix, Fig. S4 D and E*). Hence NSF_{RAN07} binding is sensitive to the α -SNAP C-terminal residues that are polymorphic in the *Rhg1* resistance-type α -SNAPs.

We then examined whether binding to *Rhg1* α -SNAPs is influenced by two of the key NSF_{RAN07} polymorphisms (Y₂₁ and F₁₁₆) that are near predicted α -SNAP binding patches in the 3D model. We restored these two residues back to the identities in WT NSF_{Ch07}, while retaining the other three NSF_{RAN07} polymorphisms (Q₄, N₂₅, and I₁₈₁). Performing in vitro binding assays as above, we observed a reduced ability of NSF_{RAN07} Y₂₁N F₁₁₆ \wedge , compared with unaltered NSF_{RAN07}, to bind resistance-type α -SNAPs (Fig. 2F and *SI Appendix, Fig. S4E*). Mutating these

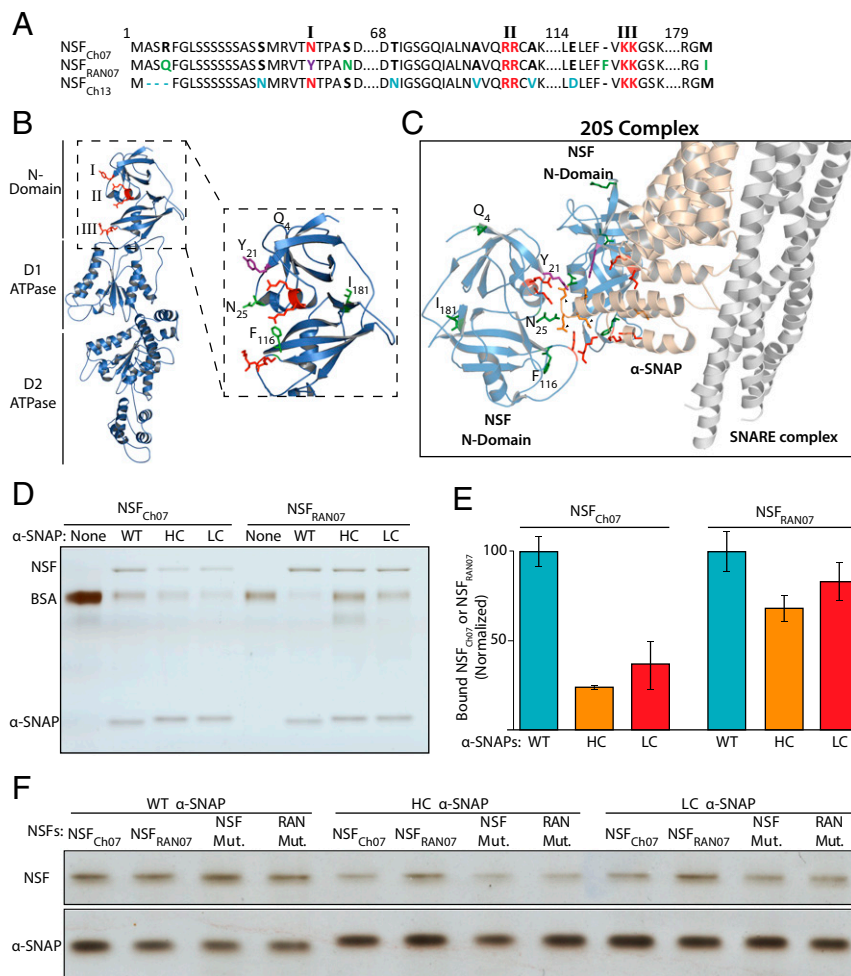


Fig. 2. *Rhg1*-containing lines carry an *NSF_{Ch07}* allele (*RAN07*) with N-domain polymorphisms at the α -SNAP binding interface that enhance binding with polymorphic *Rhg1* resistance-type α -SNAPs. (A) Alignment of N-terminal domains of soybean *NSF_{Ch07}*, *NSF_{Ch13}*, and *NSF_{RAN07}*. Large identical regions are omitted. N-domain residues corresponding to those that bind α -SNAP are colored red (*N₂₁*, *RR₈₂₋₈₃*, *KK₁₁₇₋₁₁₈*). *NSF_{RAN07}* polymorphisms *R₄Q*, *S₂₅N*, *I₁₁₆F*, and *M₁₈₁I* are colored green or purple (*N₂₁Y*); unique *NSF_{Ch13}* residues are colored light blue. (B) *NSF_{RAN07}* modeled to *NSF_{CHO}* cryo-EM structure (3J97A, State II). NSF residue patches implicated in α -SNAP binding are colored red and labeled I, II, or III. Zoomed-in view shows *NSF_{RAN07}* N-domain polymorphisms colored green or purple (*N₂₁Y*). (C) Cryo-EM structure of mammalian 20S supercomplex, masked to show only SNARE bundle (white), one α -SNAP (yellow), and two NSF N domains (light blue). Shown are the mammalian residues; conserved NSF N-domain patches (I, *R₁₀*; II, *RK₆₇₋₆₈*; III, *KK₁₀₄₋₁₀₅*) are shown in red, and α -SNAP C-terminal contacts (*D₂₁₇DEED₂₉₀₋₂₉₃*) are shown in orange. Black arrowheads point to three orange α -SNAP residues *EED₂₉₁₋₂₉₃* corresponding to sites of C-terminal polymorphisms in α -SNAP_{*Rhg1*}HC and α -SNAP_{*Rhg1*}LC. *NSF_{RAN07}* polymorphism sites are colored green, except *N₂₁Y* is in purple. (D) Silver-stained SDS/PAGE showing amount of recombinant *NSF_{Ch07}* or *NSF_{RAN07}* bound in vitro by a fixed quantity of the recombinant α -SNAP protein indicated on second line: no- α -SNAP control (None) or WT, LC or HC *Rhg1* α -SNAP. (E) Densitometric quantification of *NSF_{Ch07}* or *NSF_{RAN07}* bound as in D by the *Rhg1* α -SNAPs denoted at bottom; data are from three independent experiments, and error bars show SEM. (F) Like D, but showing recombinant *NSF_{Ch07}*, *NSF_{RAN07}*, or mutants of either, bound in vitro by *Rhg1* α -SNAPs. NSF Mut. and RAN Mut. refer to *NSF_{Ch07}* *N₂₁A F₁₁₅A* and *NSF_{RAN07}* *Y₂₁N F₁₁₆A*, respectively.

two positions to alanine in an otherwise WT *NSF_{Ch07}* (*NSF_{Ch07}* *N₂₁A F₁₁₅A*) did not restrict binding with WT α -SNAP, and binding of this *NSF_{Ch07}* *N₂₁A F₁₁₅A* with either α -SNAP_{*Rhg1*}HC or α -SNAP_{*Rhg1*}LC was still impaired (Fig. 2F and SI Appendix, Fig. S4D). Combined, these in vitro binding results suggest that *NSF_{RAN07}* not only maintains normal binding with WT α -SNAPs but can also accommodate the unusual C-terminal polymorphisms of the *Rhg1* resistance-type α -SNAPs.

The *NSF_{RAN07}* Polymorphisms Guard Against the Cell Death Induced by *Rhg1* Resistance-Type α -SNAPs. We previously observed that transient expression of either α -SNAP_{*Rhg1*}HC or α -SNAP_{*Rhg1*}LC in *N. benthamiana* leaves, via *Agrobacterium* infiltration, is cytotoxic and elicits a hyperaccumulation of the endogenous NSF protein (17). Coexpression of a WT α -SNAP with the *Rhg1* resistance-type α -SNAP diminishes this toxicity in a dose-dependent manner, and also relieves negative impacts on sec-GFP secretion (17). The

penultimate amino acid (conserved leucine) of α -SNAP, which has been implicated in stimulation of NSF ATPase, is needed for rescue of this *N. benthamiana* cytotoxicity (17, 20, 51). We subsequently conducted site-directed mutagenesis experiments which provided further evidence that the *N. benthamiana* assay closely correlates with known α -SNAP/NSF behaviors. In a first set of replicated studies, the toxicity of *Rhg1* α -SNAP expression and the capacity of coexpressed WT α -SNAP to protect against *Rhg1* α -SNAP toxicity were both observed to be dependent on intact SNARE-binding sites within the respective α -SNAPs (SI Appendix, Fig. S5).

We then examined whether, like WT α -SNAP, coexpression of soybean NSF might alleviate the toxicity of *Rhg1* resistance-type α -SNAPs in *N. benthamiana*. Similar to ref. 17, mixed *Agrobacterium* inocula were used, with ratios varying from 1:4 (one part NSF-expressing strain to four parts α -SNAP_{*Rhg1*}LC-expressing strain) all the way down to 1:19. NSF coexpression strongly reduced *Rhg1* α -SNAP cytotoxicity (Fig. 3 and SI Appendix, Fig. S6).

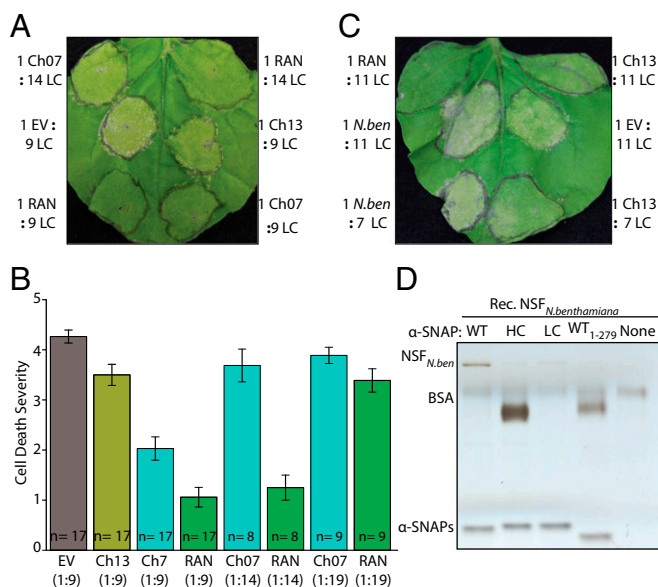


Fig. 3. Coexpression of soybean NSFs reduces cell death symptoms caused by α -SNAP_{Rhg1}LC; NSF_{RAN07} gives strongest protection. (A) *N. benthamiana* leaves ~6 d after agroinfiltration with 9:1 or 14:1 strain mixture (9 or 14 parts *Agrobacterium* that delivers LC [α -SNAP_{Rhg1}LC] to one part *Agrobacterium* that delivers the indicated soybean NSF [NSF_{Ch07}, NSF_{RAN07}, NSF_{Ch13}] or EV control). (B) Scoring of cell death severity, across multiple independent experiments, in *N. benthamiana* leaf patches coexpressing NSF_{Ch07}, NSF_{RAN07}, or NSF_{Ch13}; n is number of leaves scored; error bars show SEM. (C) Like A, but 7:1 or 11:1 mixed cultures expressing α -SNAP_{Rhg1}LC with NSF_{RAN07}, NSF_{N.benth}, NSF_{Ch13}, or empty vector. (D) Silver-stained SDS/PAGE of recombinant NSF_{N.benth} bound in vitro by recombinant WT, LC, or HC *Rhg1* α -SNAP proteins or WT α -SNAP lacking the final 10 C-terminal residues (WT₁₋₂₇₉).

No macroscopic phenotypes indicative of stress were observed upon expressing NSF_{RAN07} or NSF_{Ch07} alone (SI Appendix, Fig. S6A). Titration of the dose–response for NSF-expressing *Agrobacterium* strains identified a range of effective strain ratios (Fig. 3B). We observed that coexpressing soybean NSF_{Ch07}, NSF_{Ch13}, or NSF_{RAN07} reduced cell death caused by α -SNAP_{Rhg1}LC compared with empty vector controls (Fig. 3A and B). However, NSF_{RAN07} coexpression consistently conferred greater protection than either NSF_{Ch07} or NSF_{Ch13} (Fig. 3A and B). Across multiple independent sets of leaves tested at a variety of ratios, we observed that leaf patches coinfiltrated with NSF_{RAN07} exhibited less cell death and/or slower death. Both NSF_{RAN07} and NSF_{Ch07} were more effective than NSF_{Ch13} at rescuing cell death (Fig. 3A and B and SI Appendix, Fig. S6B). Protection against α -SNAP_{Rhg1}HC-induced cell death with NSF_{RAN07} vs. NSF_{Ch07} produced similar results (SI Appendix, Fig. S6B).

As noted above, we have consistently observed elevated abundance of the endogenous *N. benthamiana* NSF (NSF_{N.benth}) upon expression of *Rhg1* resistance-type α -SNAPs, yet this does not prevent cell death (17) (Fig. 1). However, it was unclear whether immediate coexpression of NSF_{N.benth} (81% identity to soybean NSF_{Ch07}; see SI Appendix, Fig. S7 for alignment) might lessen the cytotoxicity. Therefore, we agroinfiltrated mixed cultures expressing NSF_{N.benth} and α -SNAP_{Rhg1}LC, as well as empty vector, NSF_{Ch13}, and NSF_{RAN07} as controls. As in Fig. 3A, NSF_{Ch13} gave partial protection while NSF_{RAN07} coexpression gave strong protection (Fig. 3C). NSF_{N.benth} coexpression, on the other hand, was similar to empty vector controls and did not guard against α -SNAP_{Rhg1}LC-induced cell death (Fig. 3C). Because no obvious cell death rescue from coexpressing NSF_{N.benth} was apparent, we also examined NSF_{N.benth} physical binding with *Rhg1* resistance-type α -SNAPs, using recombinant NSF_{N.benth} protein. NSF_{N.benth} readily bound

α -SNAP_{Rhg1}WT, but binding to either *Rhg1* resistance-type α -SNAP was much lower, only slightly over negative controls (Fig. 3D). These experiments suggest that NSF_{N.benth} exhibits little or no functional interaction with SCN resistance-associated soybean *Rhg1* α -SNAPs, which likely accounts for the high toxicity of *Rhg1* α -SNAPs in *N. benthamiana*.

We then used the *N. benthamiana* assay to examine NSF_{RAN07} function predictions. One set of experiments tested whether cell death caused by α -SNAP_{Rhg1}LC₁₋₂₇₉, which lacks the final 10 C-terminal residues, could be rescued by NSF_{RAN07} or NSF_{Ch07}. Neither NSF_{RAN07} nor NSF_{Ch07} prevented the cell death caused by α -SNAP_{Rhg1}LC₁₋₂₇₉, despite guarding against cell death in the positive control treatments involving full-length α -SNAP_{Rhg1}LC (SI Appendix, Fig. S8A). Likewise, we tested whether cell death caused by α -SNAP_{Rhg1}LC 286_{AAAA}289—which also did not exhibit in vitro binding of NSF—could be rescued by either NSF_{RAN07} or NSF_{Ch07}. The α -SNAP_{Rhg1}LC 286_{AAAA}289, like α -SNAP_{Rhg1}LC, elicited increased expression of the endogenous *N. benthamiana* NSF (SI Appendix, Fig. S8B and C). However, compared with α -SNAP_{Rhg1}LC, which does bind the tested soybean NSF to some extent, we observed that α -SNAP_{Rhg1}LC 286_{AAAA}289-induced cell death was not strongly protected by NSF_{RAN07} or NSF_{Ch07} coexpression (SI Appendix, Fig. S8B). These experiments provide further evidence that C-terminally mutagenized α -SNAPs can disrupt the function of *N. benthamiana* 20S complexes, and that NSF rescue of the cell death induced by toxic α -SNAPs requires an intact C terminus of α -SNAPs to mediate successful α -SNAP–NSF interaction.

Turning to the NSFs mutagenized at the inferred α -SNAP binding interface, α -SNAP_{Rhg1}LC cell death rescue via coexpression of mutated NSF_{Ch07} or NSF_{RAN07} (NSF_{Ch07} N₂₁A F₁₁₅A or NSF_{RAN07} Y₂₁A F₁₁₆) was not as robust as rescue by the normal NSF_{Ch07} or NSF_{RAN07} (SI Appendix, Fig. S8F and G). Anti-NSF immunoblots confirmed the expression of NSF_{Ch07}, NSF_{RAN07}, and their respective mutants (SI Appendix, Fig. S8E). This supports the contribution of the mutated NSF residues to optimal NSF/ α -SNAP interaction.

Finally, we made and used an α -SNAP_{Rhg1}LC I₂₈₉A to examine how the penultimate α -SNAP residue, which has been shown in other α -SNAPs to help stimulate NSF ATPase, affected rescue by NSF_{RAN07} or NSF_{Ch07} (20, 49). Protection against α -SNAP_{Rhg1}LC I₂₈₉A was evident but was much less than that observed for α -SNAP_{Rhg1}LC (SI Appendix, Fig. S8D), suggesting that although NSF_{RAN07} may bind *Rhg1* resistance-type α -SNAPs more effectively, ATPase stimulation is likely an additional factor in relieving cytotoxicity. Overall, the findings of Fig. 3 extend the Fig. 2 finding that NSF_{RAN07} binds *Rhg1* α -SNAPs better, demonstrating in vivo that the NSF_{RAN07} polymorphisms more effectively guard against the disruptive effects of the polymorphic *Rhg1* α -SNAPs, and demonstrating that, among site-directed mutants, the extent of this in planta protection correlates with observed in vitro α -SNAP–NSF binding differences.

One Hundred Percent of Predicted *Rhg1*⁺ *Glycine max* Accessions in the US Department of Agriculture Soybean Collection Contain the NSF_{RAN07} R₄Q Amino Acid Polymorphism. NSF_{RAN07} was present in all *Rhg1*-containing -type and NAM lines (SI Appendix, Tables S1 and S2), but we sought to test whether this *Rhg1*/NSF_{RAN07} association was universal rather than “frequent.” In 2015, Song and coworkers (52–54) reported genotyping the US Department of Agriculture (USDA) soybean germplasm collection of ~20,000 accessions—collected from over 80 countries—using a 50,000 SNP DNA microarray chip (SoySNP50K iSelect BeadChip). The data are available in a searchable SNP database at Soybase (<https://soybase.org/snps/>). Using this Soybase SNP browser, we found that a C/T SNP (ss715597431, Gm07:36,449,014) causes the NSF_{RAN07} R₄Q polymorphism. Analyzing all 19,645 USDA *Glycine max* accessions for ss715597431, we estimated the NSF_{RAN07} allele frequency in the

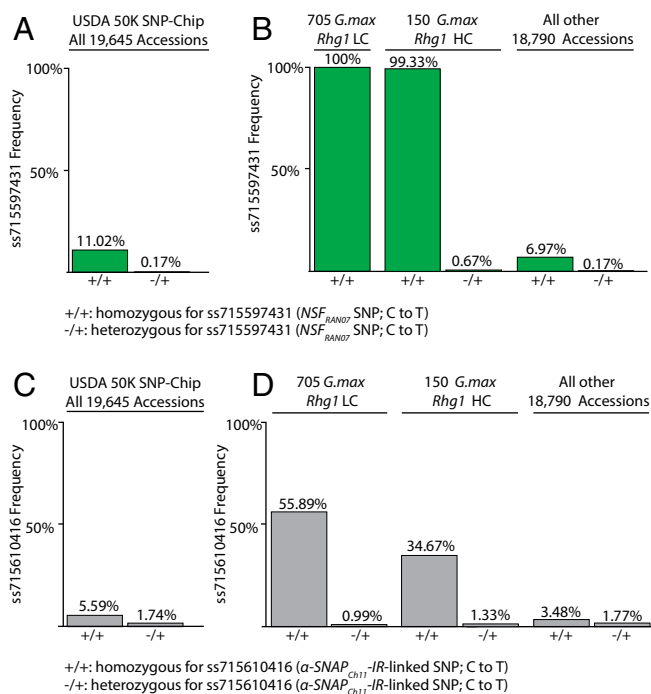


Fig. 4. All soybeans in the USDA germplasm collection that carry an *Rhg1*⁺ SNP signature also carry the R₄Q *NSF*_{RAN07} polymorphism. (A) Frequency of SoySNP50K SNP ss715597431 (corresponding to *NSF*_{RAN07} R₄Q) in all 19,645 SoySNP50K-genotyped *Glycine max* accessions. (B) Frequency of ss715597431 in all USDA collection *G. max* with *Rhg1*_{LC} or *Rhg1*_{HC} haplotype signatures, or in the remainder of SoySNP50K-genotyped *G. max*. (C) Frequency of SoySNP50K SNP ss715610416 that is closest marker for α -SNAP_{Ch11-IR} allele, across all 19,645 genotyped USDA *G. max* accessions. (D) Frequency of ss715610416 in all USDA collection *G. max* with *Rhg1*_{LC} or *Rhg1*_{HC} haplotype signatures, or in remainder of SoySNP50K-genotyped *G. max*.

USDA collection at 11.0% (2,165 *NSF*_{RAN07}⁺/*NSF*_{RAN07}⁺, 33 *NSF*_{RAN07}⁺/*NSF*_{RAN07}⁻) (Fig. 4A). Q₄ was not found in the predicted NSF protein sequences of any plant species available for query at Phytozome.org (55) (SI Appendix, Fig. S9).

Rhg1-mediated SCN resistance is uncommon among soybean accessions, and less than 5% of the USDA soybean collection carries a multicopy *Rhg1* haplotype. Previously, Lee et al. (11) identified SoySNP50K signatures for *Rhg1*_{HC}, *Rhg1*_{LC}, and single-copy (SCN-susceptible) haplotypes, and estimated that 705 *Rhg1*_{LC} and 150 *Rhg1*_{HC} accessions were present in the USDA *Glycine max* collection. Among these 855 *Rhg1*-signature *Glycine max* accessions, we determined a 100% incidence of the ss715597431 *NSF*_{RAN07} signature (Fig. 4B).

To better define the *Rhg1*-cosegregating locus within the Ch07 interval, we examined amino acid changes within candidate loci adjacent to *NSF*_{RAN07} from *Rhg1*-carrying and NAM lines, between markers ss715597415 and ss715597431. We observed that the *NSF*_{RAN07} SNPs, especially those causing the five polymorphisms in the N domain, were 100% maintained across all *Rhg1*-containing varieties. On the other hand, SNPs causing amino acid changes within candidate loci adjacent to *NSF*_{RAN07} were not 100% conserved across all *Rhg1*-containing varieties (SI Appendix, Table S3). The predicted amino acid sequence of most candidate loci matched the Wm82 (SCN-susceptible) sequence. Among candidate loci with amino acid substitutions, including *Glyma.07g196000* and *Glyma.07g196200* flanking *NSF*_{Ch07}/*Glyma.07g195900* on the side not described in SI Appendix, Table S3, only *NSF*_{RAN07} encoded the same consistent amino acid changes across all examined *Rhg1*-containing germplasm.

An SNP Associated with the Ch11 α -SNAP Intron Retention Allele—A Predicted SCN Resistance Quantitative Trait Locus—Is also Enriched Among Predicted *Rhg1*⁺ Accessions in the USDA Collection. A recent study implicated the interval carrying the intron retention allele of α -SNAP_{Ch11} (α -SNAP_{Ch11-IR}) in SCN resistance, but the responsible gene(s) within this quantitative trait locus (QTL) interval were not defined (38). The α -SNAP_{Ch11-IR} allele may have emerged randomly or it may confer some selective advantage, for example, by reducing available levels of WT α -SNAP proteins and shifting the balance toward the disruptive *Rhg1* α -SNAP proteins. This could be particularly relevant in *Rhg1*_{LC} soybean lines that typically carry only three copies of α -SNAP_{Rhg1}LC with correspondingly lower mRNA abundance, in contrast to the 9- or 10-copy *Rhg1*_{HC} lines (10, 13). We therefore used SoySNP50K data to analyze the frequency of the α -SNAP_{Ch11-IR} allele in the whole USDA collection and in the 855 *Rhg1*⁺ *Glycine max* accessions noted in the preceding section of Results. We found a C/T SNP (ss715610416, Gm11:32951515) located ~17,000 bp downstream of the α -SNAP_{Ch11} locus that was associated with the α -SNAP_{Ch11-IR} allele, as indicated by our WGS data. Using immunoblots, we tested for total levels of WT α -SNAP protein among several *Rhg1*_{LC} accessions that had either WT or α -SNAP_{Ch11-IR}-associated SNPs (ss715610416). The *Rhg1*_{LC} accessions possessing the WT-linked SNP had higher WT α -SNAP abundance relative to the *Rhg1*_{LC} accessions with the ss715610416 SNP (SI Appendix, Fig. S10). Across the USDA soybean collection, we then found that the α -SNAP_{Ch11-IR}-associated ss715610416 genotype was present in 5.6% of accessions (Fig. 4C). Perhaps surprisingly, we observed the α -SNAP_{Ch11-IR}-associated ss715610416 genotype in only half (55.9%) of the *Rhg1*_{LC} soybean lines and in about a third (34.7%) of the *Rhg1*_{HC} lines (Fig. 4D). However, this enrichment of the α -SNAP_{Ch11-IR}-linked SNP within *Rhg1*⁺ germplasm provides further evidence that this allele beneficially contributes to *Rhg1*⁺ soybean varieties.

All *Rhg1*⁺ Recombinant Inbred Lines Derived from *Rhg1*⁺ x *rhg1*⁻ Crosses Inherit *NSF*_{RAN07}. Our findings regarding *NSF*_{RAN07} co-occurrence with *Rhg1* in the USDA soybean germplasm collection are an indication of strong segregation distortion. However, recalling that Webb et al. (39) reported that only 91 of 96 lines with a resistant parent marker type linked to *Rhg1* also had a resistant parent marker type near the QTL now known to encode *NSF*_{RAN07}, we explored whether the *Rhg1*⁺ progeny of more recent biparental crosses strictly inherited *NSF*_{RAN07}. From the SoyNAM project (47), we examined F5 genotypic data for populations of derived recombinant inbred lines (RILs) developed from crosses of the IA3023 (SCN-susceptible) hub parent to eight different soybean accessions carrying either *Rhg1*_{HC} (seven accessions) or *Rhg1*_{LC} (one accession). There were 122 to 139 RILs in each population. The segregation for *NSF*_{RAN07}:*NSF*_{Ch07}WT in soybean lines lacking *Rhg1* did not deviate from the null hypothesis of 1:1 segregation in six of the eight populations. The segregation distortion for *NSF*_{RAN07} was obvious among RILs that carried a resistance-associated *Rhg1* allele, but, out of a total of 309 *Rhg1*⁺ RILs, 8 appeared to have inherited *Rhg1*_{HC} or *Rhg1*_{LC} and a WT *NSF*_{Ch07} allele (SI Appendix, Table S4). This was based upon the low-density SoySNP6K mapping data that used linked rather than perfect genetic markers for *Rhg1* and *NSF* (47). We therefore genotyped all eight of these RILs via sequencing and/or primers detecting the *Rhg1* repeat junction and a WT *NSF*_{Ch07} vs. *NSF*_{RAN07} allele and found that all eight reexamined RILs containing *Rhg1*_{HC} or *Rhg1*_{LC} also carried the *NSF*_{RAN07}^{116F} and M_{181I} mutations. Thus, all *Rhg1*⁺ RILs also inherited *NSF*_{RAN07} (SI Appendix, Table S4). We analogously infer that the five lines of the Webb et al. (39) study that appeared to break coinheritance between *Rhg1*_{HC} and *NSF*_{RAN07} likely underwent a cross-over between the

gene in question and the genetic markers linked to either *Rhg1* or *NSF*. Taken together with the described biochemical and *in planta* impacts of *Rhg1* α -SNAPs and NSF_{RAN07}, the SoySNP50K and NAM data indicate that NSF_{RAN07} coinheritance is a necessary balance that confers viability to soybeans carrying a resistance-type *Rhg1* haplotype.

Discussion

Across eukaryotes, NSF and α -SNAP interact through conserved electrostatic contacts to disassemble SNARE complexes, thereby maintaining cellular vesicle trafficking (14, 15). Our findings indicate that *Rhg1*-mediated SCN resistance in soybean encompasses not just unusual changes in *Rhg1* α -SNAP sequence and abundance in syncytium cells, as previously published, but also changes in other housekeeping α -SNAP and NSF genes whose products comprise the SNARE recycling machinery. These results showcase how a functionally related set of multiple conserved housekeeping genes has coevolved toward atypical forms, apparently to confer resistance to a highly damaging nematode pathogen while balancing plant fitness. The findings suggest that the two common resistance-conferring *Rhg1* haplotypes employ similar yet distinct strategies to combat SCN: They decrease WT α -SNAP availability via greater *Rhg1* copy number expansion and/or through loss of WT α -SNAP loci. We also found that presence of the unusual *Rhg1* α -SNAP proteins requires copresence of a novel NSF protein for plant viability. This explains a well-documented segregation distortion occurring between *Rhg1* and a chromosome 07 region (39–41). Perhaps more importantly, this study and other recent work on *Rhg1* offer a molecular framework in which to understand the interactions of multiple QTLs associated with SCN resistance (13, 17, 18, 38, 56, 57): Many of these loci modify the host vesicle fusion SNARE recycling machinery as a means of controlling SCN infection.

An understanding of the necessity of NSF_{RAN07} to balance *Rhg1* germplasm should become a central consideration in any planned transgenic addition of *Rhg1* into SCN-susceptible soybeans. Beyond soybean, this report suggests strategies to engineer *Rhg1*-like resistance into other cyst nematode-susceptible crop species, through introduction of sequence-edited α -SNAP alleles together with modulation of WT α -SNAP abundance and/or introduction of a compatible NSF.

It is biologically fascinating that complementary α -SNAP and NSF polymorphisms, located at the conserved binding interfaces of both members of the core SNARE recycling machinery, were apparently selected due to disease pressure from SCN. It highlights this pathway's importance during the pathogen–host interaction. The previous finding that *Rhg1* resistance-type α -SNAPs are impaired in normal NSF interactions (17) is supported by the present finding that a unique NSF allele—NSF_{RAN07}—is a requisite balance for *Rhg1* resistance-type α -SNAPs. While ref. 17 proposed the functional redundancy of multiple WT α -SNAP loci (available due to polyploidy) as the balance that allows the viability of *Rhg1*-containing lines, this model must be modified with the observation that *Rhg1*-containing lines that lack NSF_{RAN07} are apparently nonviable. Presence of WT α -SNAPs may still, in the presence of NSF_{RAN07}, contribute to the vigor and normal soybean yield of lines carrying the PI 88788 source of *Rhg1* (*Rhg1_{HC}*), but they are not sufficient to do so in the absence of NSF_{RAN07}.

Housekeeping genes have been reported to evolve particularly slowly due to selective constraints (58), which raises interest in the coevolution between NSF_{RAN07} and *Rhg1* α -SNAP. It is unclear whether existing natural variation at Ch07 NSF among soybean populations enabled the development of the Ch18 *Rhg1* resistance-type α -SNAPs or vice versa, or if the *Rhg1* α -SNAP duplication event occurred first, followed by subsequent coevolution of NSF and resistance-type α -SNAP polymorphisms. Currently, reports of natural NSF variation appear to be limited to humans. The 1,000 Human Genomes Project showed that, in

certain human ethnicities, NSF copy number expansions of up to three repeats are not uncommon (59). The original NSF locus is full length, while the subsequent NSF copy number repeats truncate near exon 13 and do not encode full-length NSF transcripts (59, 60). A recent study reported a correlation between this human NSF copy number variation and drug dependency (60). Notably, no residue substitutions were reported among human NSF alleles, and, to the best of our knowledge, no naturally occurring NSF protein variants from any organism have previously been reported.

As noted above, our findings about NSF_{RAN07} provide a mechanistic explanation for the previously observed segregation distortion, in SCN-resistant plants, between *Rhg1* and the chromosome 07 genetic interval that encodes NSF_{RAN07} (39–41). An observation that remains less firmly explained, however, is why transgenic expression of α -SNAP_{Rhg1_{HC}} or α -SNAP_{Rhg1_{LC}} protein, in *Agrobacterium rhizogenes*-transformed root systems of SCN-susceptible Wm82 (which lacks NSF_{RAN07}), elicited no apparent sensitivities such as cytotoxicity or endogenous NSF expression increases (10, 17). These sensitivities were observed with *N. benthamiana* expressing *Rhg1* α -SNAPs (17). Notably, coexpression of NSF_{N.benth} did not relieve the cell death in *N. benthamiana* leaves caused by *Rhg1* α -SNAPs, while WT soybean NSF_{Ch07} did, albeit not as effectively as NSF_{RAN07}. Consistent with this, recombinant NSF_{N.benth} essentially could not bind with *Rhg1* resistance-type α -SNAPs *in vitro*, but those α -SNAPs could bind soybean WT NSF_{Ch07}. This may explain why soybean root cells do exhibit some tolerance of *Rhg1* α -SNAP expression even in the absence of NSF_{RAN07}. Nevertheless, the finding that all soybeans in the USDA collection that bear the signature of resistance-conferring *Rhg1* alleles also contain the NSF_{RAN07} R₄Q signature, coupled with the universal copresence of the NSF_{RAN07} allele with *Rhg1* in the segregating progeny of NAM crosses, provides compelling evidence that, at the organismal level, NSF_{RAN07} is essential for viability at some stage of growth for all *Rhg1*-containing germplasm.

Rhg1_{LC} and *Rhg4* contribute together to the SCN resistance of *Rhg1_{LC}* soybean lines (4, 61), and it remains unclear why *Rhg1_{LC}* confers only partial SCN resistance when *Rhg4*, which encodes a putative serine hydroxymethyltransferase, is absent (61–63). Whether or not the *Rhg4* product directly impacts *Rhg1*-associated α -SNAP/NSF/SNARE interactions, consideration of the present findings may be influenced by published evidence that *Rhg1_{HC}* soybean lines are substantially more effective than *Rhg1_{LC}⁺ rhg4⁻* lines at conferring SCN resistance against HG type 0 SCN populations (62, 63).

The present findings add to what was already known or inferred about loss of some WT α -SNAPs in Peking-type *Rhg1_{LC}* soybean lines (11, 13, 37, 38). *Rhg1_{LC}* varieties without or with the α -SNAP_{Ch11-IR} allele exhibit reduced or sharply reduced WT α -SNAP expression, respectively. This further supports the idea that, in addition to the unusual *Rhg1* α -SNAP proteins, WT α -SNAP levels and the [WT α -SNAP:*Rhg1* α -SNAP] ratio can be important determinants of successful *Rhg1*-mediated SCN resistance. Models for resistance involving evasion of nematode effectors should also be considered. NSF_{RAN07} may have allowed the nontoxic presence of *Rhg1*-type α -SNAPs, and *Rhg1* α -SNAPs may confer SCN resistance by failing to cooperate with nematode manipulation of the host. This model could explain why divergence of *Rhg1* α -SNAP types has occurred: Different SCN populations may carry effectors that manipulate or interact with the host SNARE recycling machinery, but to varying degrees depending on the α -SNAP protein that is present.

The α -SNAP_{Ch11-IR}-associated SNP, which correlated with modest changes in WT α -SNAP abundance, was present in only about half of the *Rhg1_{LC}* soybean lines and a third of the *Rhg1_{HC}* lines. Only a subtle positive impact on SCN resistance was reported for the broader QTL locus carrying the α -SNAP_{Ch11-IR}

allele (38). However, because not all *Rhg1*⁺ soybean lines carry the α -SNAP_{Ch11-IR}-associated genotype, its intentional use or exclusion may, in the future, translate to subtle but economically useful shifts in SCN resistance, in the HG type specificity of that resistance, or in soybean yield potential.

Discovery of the need for NSF_{RAN07} in *Rhg1*-containing soybeans may reveal a protective mechanism that reduces the toxic effects of *Rhg1* α -SNAPs in some cell types/conditions by facilitating participation of *Rhg1* α -SNAPs in productive 20S complexes that disassemble SNARE bundles, while the toxicity of *Rhg1* α -SNAPs remains predominant in syncytium cells. Such conditionally functional NSF mutants are known in the laboratory-derived *Drosophila* NSF *comatose* mutants, whereby the NSF-1 protein encoded by the *comatose* allele supports SNARE complex disassembly at room temperature but is nonfunctional at elevated temperatures, leading to failure of synaptic vesicle transport and fly paralysis (31, 64). However, other mechanistic hypotheses are viable. Future studies could examine the dynamics of NSF_{RAN07} abundance and function over time in developing SCN syncytia. For example, increased NSF levels were detected in syncytia-containing root segments in *Rhg1*_{HC} varieties, and we had associated this with WT α -SNAP deficiency (17), but whether it is NSF_{RAN07} or NSF_{Ch13} that increases is of interest and might suggest whether α -SNAP and NSF functionality is being promoted or disrupted by the host. We did observe that NSF_{RAN07} apparently can work with WT α -SNAPs, or at least is not toxic in the way that resistance-associated *Rhg1* α -SNAPs can be toxic. Expression of NSF_{RAN07} in *N. benthamiana* caused no macroscopically detectable leaf phenotypes, and NSF_{RAN07} is expressed in *Rhg1*_{HC} soybeans that also express high levels of WT α -SNAPs. The random segregation of the alleles encoding NSF_{Ch07}WT and NSF_{RAN07} in soybean progeny that lack *Rhg1*, and the presence of NSF_{RAN07} in over 1,300 USDA soybean accessions that lack *Rhg1*, also suggests that NSF_{RAN07} likely functions effectively with WT α -SNAPs.

A summarizing model can be constructed. We hypothesize that coexpression of WT α -SNAPs or soybean NSFs can compete away the toxicity of *Rhg1* α -SNAPs by restoring functionally compatible partners to the 20S complex. The α -SNAPs bind bundles of three or four SNARE proteins and provide a key portion of the platform for binding of NSF proteins and the stimulation of ATP hydrolysis to disassemble those SNARE bundles. The success of the α -SNAP *N. benthamiana* toxicity assay apparently derives from the inability of NSF_{N.benth} to function on SNARE bundles that carry *Rhg1* α -SNAPs. The phenotype caused by *Rhg1* α -SNAP expression is extreme in *N. benthamiana* but mild in most soybean cells because of the partial compatibility of *Rhg1* α -SNAPs with WT soybean NSFs. Our data indicate that even greater compatibility with *Rhg1* α -SNAPs is restored by presence of NSF_{RAN07}. Nevertheless, the findings of the present work and ref. 17 indicate that *Rhg1* α -SNAPs are a less compatible partner than WT α -SNAPs. When the relative level of *Rhg1* α -SNAPs goes up, as has been documented for syncytium cells (17), we hypothesize that the suboptimal function of *Rhg1* α -SNAPs poisons syncytia. Alternative models for SCN resistance are possible; for example, the *Rhg1* α -SNAPs may be less sensitive to SCN effectors that manipulate WT α -SNAPs to the advantage of the nematode. In either case, we propose that NSF_{RAN07} is sufficiently compatible with *Rhg1* α -SNAPs to confer viability and productivity to *Rhg1*⁺ soybean lines, especially when WT α -SNAPs are also abundant. NSF_{RAN07} may not be sufficient to overcome the toxicity of *Rhg1* α -SNAPs in syncytia. The lower abundance of WT α -SNAPs in many *Rhg1*_{LC} lines may be important to enhancing the SCN resistance of those lines, where there are only 3 rather than ~10 tandem repeat copies of *Rhg1*, but it may also be a primary reason why *Rhg1*_{LC} lines have been widely observed to exhibit minor reductions in grain yield.

The amassing evidence for the importance of altered α -SNAP/NSF/SNARE interactions in SCN–soybean interactions also suggests that these proteins may be attractive targets for cyst nematode effectors (13, 17, 18, 38, 56, 57, 65). Preliminary evidence for one such effector is already in place (57), and extensive variation is present in the SCN genes that encode putative SNARE-like protein effectors (66). The gradual evolution of SCN populations toward an increasing number of individuals that can overcome the widely used *Rhg1*_{HC} SCN resistance is a major issue for global soybean production (67). Future work to discover and understand relevant nematode effectors in these SCN populations, and a means of reestablishing resistance against such nematodes, may benefit from assays that directly test for effectors that impact the soybean α -SNAP and NSF protein variants characterized in the present study.

Materials and Methods

Antibodies and Immunoblotting. Affinity-purified polyclonal rabbit antibodies raised against peptides from soybean NSF, α -SNAP_{Rhg1}HC, α -SNAP_{Rhg1}LC, and WT α -SNAPs were previously generated and validated using recombinant proteins as described in ref. 17. Tissue preparation and immunoblots were performed essentially as in refs. 17 and 68.

Transgenic Soybean Root Generation. Binary expression constructs were transformed into *A. rhizogenes* strain "ARqua1," and transgenic soybean roots were produced from cotyledons of the noted genetic background as described in ref. 10.

Recombinant Protein Production. Soluble, native recombinant His-SUMO- α -SNAP or His-SUMO-NSF proteins were expressed and purified by similar procedures as described in ref. 17.

In Vitro α -SNAP NSF Binding Assays. In vitro binding assays were performed with recombinant α -SNAP and NSF proteins essentially as described in refs. 17 and 49. Briefly, 20 μ g of recombinant α -SNAP was adhered to the bottom of a polypropylene tube at room temperature, and then washed. Subsequently, 20 μ g of recombinant NSF was added to each tube containing immobilized α -SNAP and allowed to bind on ice for 10 min, followed by two washes; α -SNAP and bound NSF were then collected, separated by SDS/PAGE, and visualized by silver staining.

Transient Agrobacterium Expression in *N. benthamiana*. *Agrobacterium tumefaciens* strain GV3101 was used for transient protein expression in *N. benthamiana*. Plant growth, culture induction, and infiltration were performed essentially as in ref. 17. *N. benthamiana* toxicity results were quantified using a standardized 0 to 5 lesion severity scoring system with blinded treatments (raters unaware of which treatments they are scoring). Consistency of scoring among independent raters was confirmed.

Segregating NAM Crosses. Soybean parental crosses and 6K SNP genotyping mapping are described in ref. 47.

Protein Structure Modeling. NSF_{RAN07}, α -SNAP_{Ch11}, and α -SNAP_{Ch11}IR structural homology models were generated using SWISS-MODEL (Biozentrum), and the resulting PDB files were analyzed with PyMOL (The PyMOL Molecular Graphics System, Version 1.8; Schrödinger, LLC).

DNA Sequence and SNP Analysis. WGS data of 12 soybean varieties were obtained from previously published studies and analyzed as in Cook et al. (13, 47).

Detailed information regarding the procedures used is provided in *SI Appendix, SI Materials and Methods*.

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