Arabidopsis genes, AtNPR1, AtTGA2 and AtPR-5, confer partial resistance to soybean cyst nematode (Heterodera glycines) when overexpressed in transgenic soybean roots

Benjamin F Matthews1*, Hunter Beard1, Eric Brewer1, Sara Kabir1, Margaret H MacDonald1 and Reham M Youssef1,2

Abstract

Background: Extensive studies using the model system Arabidopsis thaliana to elucidate plant defense signaling and pathway networks indicate that salicylic acid (SA) is the key hormone triggering the plant defense response against biotrophic and hemi-biotrophic pathogens, while jasmonic acid (JA) and derivatives are critical to the defense response against necrotrophic pathogens. Several reports demonstrate that SA limits nematode reproduction.

Results: Here we translate knowledge gained from studies using Arabidopsis to soybean. The ability of thirty-one Arabidopsis genes encoding important components of SA and JA synthesis and signaling in conferring resistance to soybean cyst nematode (SCN: Heterodera glycines) are investigated. We demonstrate that overexpression of three of thirty-one Arabidopsis genes in transgenic soybean roots of composite plants decreased the number of cysts formed by SCN to less than 50% of those found on control roots, namely AtNPR1 (33%), AtTGA2 (38%), and AtPR-5 (38%). Three additional Arabidopsis genes decreased the number of SCN cysts by 40% or more: AtACBP3 (53% of the control value), AtACD2 (55%), and AtCM-3 (57%). Other genes having less or no effect included AtEDS5 (77%), AtNDR1 (82%), AtEDS1 (107%), and AtPR-1 (80%), as compared to control. Overexpression of AtDND1 greatly increased susceptibility as indicated by a large increase in the number of SCN cysts (175% of control).

Conclusions: Knowledge of the pathogen defense system gained from studies of the model system, Arabidopsis, can be directly translated to soybean through direct overexpression of Arabidopsis genes. When the genes, AtNPR1, AtG2A, and AtPR-5, encoding specific components involved in SA regulation, synthesis, and signaling, are overexpressed in soybean roots, resistance to SCN is enhanced. This demonstrates functional compatibility of some Arabidopsis genes with soybean and identifies genes that may be used to engineer resistance to nematodes.

Keywords: Arabidopsis, Composite plants, Gene overexpression, Jasmonic acid, Resistance, Salicylic acid, Soybean, Soybean cyst nematode, Transgenic roots

Background

Plant parasitic nematodes cause billions of dollars in losses each year worldwide [1-3]. The root-knot nematode, genus Meloidogyne, attacks over 3000 species of plants [4,5], while the soybean cyst nematode (Heterodera glycines) has a much narrower host range and is responsible for almost one billion dollars per year in losses in the US [2,3]. Although some soybean genotypes are resistant to specific populations of SCN, no soybean genotype is known to be resistant to all SCN populations. Several genes conferring partial Resistance to Heterodera glycines (Rhg) have been mapped, and, recently, genes at the rhg1 and Rhg4 loci have been elucidated [6-9]. The defense response of soybean to SCN has been examined at the physiological, genetic, and molecular level, and several reports indicate that salicylic acid (SA), jasmonic acid
(JA), ethylene (ET), and indole acetic acid (IAA) play a role in resistance and susceptibility of plants to nematodes [8,10-15]. However, the roles of defense-related hormones and specific components of their synthesis and signaling pathways in providing resistance in plants to nematodes are unknown.

The plant defense response is complex. Plants launch a myriad of local and systemic defense responses to protect themselves from invasion by pests and pathogens. Several hormones are involved in inducing the defense response and regulating defense response networks, including SA, JA, ET, and IAA [11,12,16-18]. Plants react to pathogen-associated or microbe including SA, JA, ET, and IAA [11,12,16-18]. Plants react to pathogen-associated or microbe-associated molecular patterns (PAMPs/MAMPs) which are sensed by plants through leucine-rich repeat (LRR) receptors. PAMPs signal stomatal closure and stimulate innate immunity in plants. In general, SA activates the defense response to biotrophic and hemi-biotrophic pathogens, induces systemic acquired resistance (SAR), and triggers the expression of SAR-associated pathogenesis related genes PR-1, PR-5, and others [12,18]. The role of specific components of SA regulation, synthesis, and signaling in defending plants against parasitic nematodes is not well understood. However, SA does play a role in decreasing susceptibility to root-knot nematode (RKN) in cow pea [19] and tomato [20,21], and to the cyst nematode, Heterodera schachtii, in Arabidopsis [10]. Likewise, JA also plays a role in resistance of plants to nematodes. Foliar spraying of tomato with JA reduced galling and the final population of RKN (M. incognita); [22-26], as did pre-treatment of tomato seeds with JA [21], indicating a role for JA in plant resistance to nematodes.

Little is known of the role of specific components of SA regulation and signaling in the interaction of soybean with the soybean cyst nematode (SCN; Heterodera glycines), the major pest of soybean in the US. Although soybean genes conferring resistance to SCN have been identified, these do not provide resistance to all SCN populations. Resistance in soybean to SCN is multigenic, and several major loci for resistance have been identified [15,27-31]. For example, in soybean cv Peking, several genes (rhg1, rhg2, rhg3, and Rhg4) have been reported that confer resistance to SCN race 1 [15,32], yet none of these genes confers complete resistance to all SCN populations. Therefore, we are applying to soybean a portion of the vast knowledge that has been gained from studies on the model plant Arabidopsis and its large array of mutants on the role of SA and JA in the plant defense response to identify important components that may be useful in decreasing susceptibility of plants to nematodes, and especially of soybean to SCN.

Arabidopsis has been used widely as a model system to study plant defense pathways, usually with bacterial and fungal pathogens [11,12,17,33-39]. Much attention has been focused on SA production and signaling pathways using Arabidopsis mutants infected with bacterial and fungal pathogens (Figure 1) [12,16-18,35-44]. For example, when a virulent strain of the biotrophic pathogen, Pseudomonas syringae, attacks Arabidopsis, the AvrRPS4 effector protein of P. syringae secreted by the type III secretion system is detected by the plant receptor RPS4, a Toll-interleukin-nucleotide binding-leucine-rich repeat (TIR-NB-LRR) that mediates the induction of antimicrobial defenses to provide disease resistance. The nucleo-cytoplasmic protein ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), which is a lipase-like protein, connects RPS4 with downstream defense pathways and regulates the accumulation of SA [45,46]. EDS1 is essential for production of the hypersensitive response and mobilization of defense pathways [47-49]. EDS1 can dimerize and interact with another lipase-like protein, phytoalexin deficient 4 (PAD4) [48,49]. Both EDS1 and PAD4 are required for the accumulation of SA and they are involved in ROS signaling [46,50]. PAD4 protein is required for amplification of weak signals resulting from pathogen infection. Another important component of the defense response is EDS5, a multi-drug transporter member of the MATE family of transporters [50].

It is postulated that SA can be synthesized through two different pathways in Arabidopsis [51,52]. One pathway involves the enzyme isochorismate synthase (ICS; EC 5.4.4.2), which catalyzes the conversion of chorismate to isochorismate. The enzyme chorismate mutase (CM; EC 5.4.99.5) catalyzes the competing chemical reaction and converts chorismate to prephenate. This would divert chorismate to produce other compounds, such as phenylalanine and tyrosine. The Arabidopsis sid2-2 (SA INDUCTION-DEFICIENT 2) mutation has been mapped to the locus encompassing the ICS (SID1) gene [52]. Upon synthesis, SA can bind directly with NPR1, which is encoded by AtNPR1 (NONEXPRESSOR OF PR1), also known as NIM1 (NON-INDUCIBLE IMMUNITY 1). NPR1 is an SA receptor that is a transcriptional regulator of genes involved in the SA-dependent defense response [53], including the SA marker gene PR-1 (PATHOGENESIS RELATED 1). NPR1 interacts with transcription factor TGA2 family members, including AHBP-1b, and the complex binds to SA-responsive promoter elements of PR-1 and other SA-dependent defense genes to regulate expression [54].

SA can also be regulated independently of EDS1 and PAD4. NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE 1) is a positive regulator of SA that works independently of EDS1 and PAD4 [55] NDR1 is an integrin-like protein that can associate with RIN4, while RIN4 can associate with RPM1 and RPM2 [56]. NDR1 may play a role in electrolyte release upon infection of Arabidopsis by P. syringae, while RIN4 regulates stomatal apertures in conjunction with H+-ATPases.
of the plasma membrane of *Arabidopsis* during pathogen attack [57].

JA, JA\textsubscript{ile} and related lipid-derived compounds also act as signals in the plant defense response and are associated with resistance to necrotrophic pathogens [16,58,59]. The pathway leading to JA and JA\textsubscript{ile} synthesis and some components related to JA signaling and control are depicted in Figure 2. Allene oxide synthase (AOS) and allene oxide cyclase (AOC) are two enzymes important to JA synthesis. JAR1 encodes an ATP-dependent JA-amido synthase that...
conjugates isoleucine with JA to form JAile, which plays an essential role in JA signaling.

In this paper, we examine the role of some components of the plant defense response in conferring resistance in soybean to SCN. We show that specific Arabidopsis genes, namely AtNPR1, AtTGA2, AtICS1, and AtPR5 which encode components of SA regulation, biosynthesis, and downstream effectors, can decrease susceptibility of soybean to SCN when expressed in transgenic soybean roots. We also demonstrate that expression of Arabidopsis genes encoding AOS, AOC, and JAR1, which are involved in the synthesis and modification of JA, only modestly decrease susceptibility of soybean roots to SCN. These results indicated that some Arabidopsis genes can be directly used in soybean, thus directly applying knowledge of the defense response gained from studies using Arabidopsis as a model system to soybean to decrease susceptibility to nematodes.

Results
Expression of Arabidopsis genes in soybean roots
SA and JA are well known regulators of the plant defense response as described through studies of the model plant, Arabidopsis. To determine if some of these Arabidopsis genes could be directly used in soybean to translate knowledge from Arabidopsis to soybean, we selected and cloned genes encoding numerous components of SA and JA synthesis, regulation, and signaling from the literature describing the defense response of Arabidopsis to pathogens (Table 1). To broaden the scope of our study, we also selected several Arabidopsis genes less well defined in function or that represented other portions of the plant defense response less dependent upon SA and JA. Open reading frames (ORFs) of thirty-one Arabidopsis genes were cloned into the gene expression vector pRAP15 [8,60,61] using the primers listed in Additional file 1: Table S1. The inserted ORFs were sequenced to confirm their identity and to ensure their sequence was conserved. The vector with insert was transformed by Agrobacterium rhizogenes K599 into cells at the base of the cut stem of soybean seedlings. Approximately 35 days after transformation, untransformed roots were removed from the composite plants, and the transformed roots were inoculated with SCN.

The effect of expression of thirty-one genes on the number of SCN cysts at 35 dai (days after inoculation) was determined (Table 1). Six genes decreased the number of cysts more than 40%, thus conferring partial resistance to SCN. Three of these genes, AtNPR1, AtTGA2, and AtPR-5, decreased the number of cysts more than 60%, while three others, AtACBP3, AtACD2, and AtCMS3 decreased the number of cysts 40%. One Arabidopsis gene, AtDND1, increased the number of cysts of SCN to 175% of the control, thus making the soybean roots more susceptible to SCN (Table 1).

RNA was extracted from a subset of transformed roots for genes listed in Additional file 2: Table S2 to check for expression of the Arabidopsis gene by PCR. The amplicons were separated and visualized by gel electrophoresis and staining (Figure 3) to confirm that the ORFs were expressed in the composite root. All roots tested expressed the transcript.

In addition, the abundance of transcript of two genes providing the most protection against SCN, AtNPR1 and AtTGA2, was determined by qRT-PCR using gene specific primers (Additional file 2: Table S2). Transcript number was calculated using the sigmoidal method [62]. The number of transcripts of AtNPR1 was 40,500 molecules and for AtTGA2 was 60,500 molecules in transformed roots, while no transcripts of either gene were detectable in the control roots. In all samples, the expression level was similar for the housekeeping gene encoding ubiquitin-3 (Figure 4).

The number of transcripts of three defense-related genes, GmPR5, GmCHIB1, and GmERF1 was also determined using qRT-PCR (Figure 5). In roots overexpressing AtNPR1, there were 178 transcripts of GmPR5, while in roots overexpressing AtTGA2, there were 159 transcripts. In control roots, there were only 38 transcripts of GmPR5. Thus, GmPR5 was elevated approximately 4-fold in roots overexpressing AtTGA2. Transcripts of GmCHIB1 were also elevated in these roots. There were 403 transcripts of GmCHIB1 in roots overexpressing AtNPR1 and 133 transcripts in roots overexpressing AtTGA2. There were only 53 transcripts of GmCHIB1 in control roots. This represents an increase in expression of GmCHIB1 by approximately 8- and 2.5-fold in soybean roots overexpressing AtNPR1 and AtTGA2, respectively.

In contrasts, the number of transcripts of GmERF1 decreased in soybean roots overexpressing AtNPR1. Only 42 transcripts of GmERF1 were present, whereas control plants contained 1921 transcripts. Similarly, roots overexpressing AtTGA2 contained fewer GmERF1 transcripts than in control roots with only 75 transcripts present. Thus, GmERF1 expression was greatly decreased in both sets of transgenic roots.

SA-related genes
Activation of the defense response in plants is initiated through several parallel signaling pathways. In gene-for-gene resistance, host resistance (R) proteins indirectly recognize pathogen effectors to initiate resistance [63]. The coiled-coiled-nucleotide-binding site-leucine-rich repeat (CC-NB-LRR) and the TIR-NB-LRR classes of proteins are two major sub-groups of R protein [64]. In this study, we selected the Arabidopsis protein NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) as a representative CC-NB-LRR R-protein, because of its known role in activating the SA-mediated defense response.
in Arabidopsis. RPS2, encoded by AtRPS2, was selected as a representative of the TIR-NB-LRR class of proteins. Both AtNDR1 and AtRPS2 were overexpressed in transgenic soybean roots to determine their effect on SCN growth and maturation as measured by the female index (FI), which expresses the number of mature SCN females 35 days after root inoculation as a percent of the control value. Overexpression of AtNDR1 slightly inhibited SCN development (FI = 80), while AtRPS2 had a slightly greater inhibitory effect (FI = 71), but the effect was not statistically significant (P > 0.05) (Table 1) for either gene.

Arabidopsis NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (AtNPR1) is downstream of the R proteins NDR1 and RPS2. NPR1 is a key regulator of SAR and plays a critical role as a SA signal transducer in Arabidopsis [38,44]. When AtNPR1 was overexpressed, the FI decreased to 33% of the control. AtNPR1 had the lowest FI value among the Arabidopsis genes tested in this study (Table 1).

Alignment of the 593 aa of AtNPR1 with its closest soybean counterpart, the product of Glyma09g07440.1, indicates conservation of only 273 aa, although there are also many conservative substitutions (Figure 6). There are five soybean genes encoding proteins closely related to AtNPR1. The protein encoded by Glyma09g07440.1 is most closely related to the protein encoded by Glyma09g02430.1,
and these are closely related to the proteins encoded by Glyma15g13320.1, Glyma14g03510.1, and Glyma02g45260.1. All five soybean putative NPR1 proteins contain a BTB/POZ domain, ankyrin repeats (domain CLO465), a NPR1/NIM1-like defense protein C terminal, and a domain of unknown function (DUF3420), as does AtNPR1.

Expression of AtTGA2, encoding the TGA-type basic leucine zipper bZip transcription factor AHBP-1B, in soybean roots decreased the FI of SCN to 38% of the control (Table 1). There are numerous soybean homologs of AtTGA2. The four most highly conserved are Glyma13g26280.1, Glyma15g37220.1, Glyma20g39050.2, and Glyma10g44270.1. The amino acid sequence of AtTGA2 is closely related to Glyma20g24766.1 (5e-56; Figure 7). The alternative transcript, Glyma20g24766.2, is exactly the same, except it lacks the 5′ leader sequence. The Glyma20g24766.1 transcript appears to contain a chloroplast transit sequence as predicted using ChloroP1.1, Technical University of Denmark, http://www.cbs.dtu.dk/services/ChloroP/, while Glyma20g24766.2 does not contain this 5′ transit sequence. We used the alternate sequence Glyma20g39050.2 that lacks a 5′ leader sequence that is also missing in AtTGA2. The AtTGA2 and GmTGA2 protein sequences are highly conserved in the bZIP domain [65-68], with only two aa differences in the 39 aa domain. This region is highly conserved with only eight aa substitutions in the 65 aa region.

![Figure 3 Expression of transcripts from each gene in transformed roots.](image)

RNA was converted to cDNA and used as template for PCR amplification of a fragment of each gene. Agarose gel containing amplicons representing a portion of AtNPR1, AtTGA2, AtPR-5, AtACBP3, AtACD2, AtCM-3, AtM2, AtCDR1, and AtND1, respectively. Molecular weight markers (MWM) are shown in the first lane. Each lane represents a transgenic root from an individual plant. The cDNA from RNA extracted from wild type (WT) roots did not produce an amplicon for any of these genes. However, cDNA from the wild type was present, and an amplicon was produced by PCR when the cDNA was used as template with primers for a soybean control gene. RNA was extracted from three roots, individually, and independently made into cDNA. Each was examined by PCR and visualized as above. All samples from transgenic roots produced amplicons according to the appropriate Arabidopsis gene.

![Figure 4 Number of transcripts of AtNPR1 and AtTGA2 in transformed roots were determined in roots transformed with AtNPR1 and AtTGA2. No transcripts of either gene were detectable in the control roots. In all samples, the expression level was similar for the soybean housekeeping gene encoding ubiquitin-3 (GmUBI-3).](image)

![Figure 5 The number of transcripts of three defense-related genes, GmPR5, GmCHIB1, and GmERF1 was determined in roots transformed with (A) AtNPR1 and (B) AtTGA2 using qRT-PCR.](image)
A second domain, DOG1 [69] is found toward the carboxy terminus and is involved in the control of seed dormancy [54]. The DOG1 domain is less conserved between these Arabidopsis and soybean proteins. In Arabidopsis, SA interacts with the receptor NPR1, which then interacts with the transcription factor TGA2 to modulate the transcription of some genes, including PR-1 and PR-5. Thus, we examined the effect of overexpression of AtPR-1 and AtPR-5 on the FI of SCN. Overexpression of AtPR-1 did not have a statistically significant effect (FI = 80; P = 0.2) on the number of SCN cysts. In contrast, when AtPR-5 was overexpressed in soybean roots, the FI was decreased to 38% of the control (Table 1), while overexpression of AtPR-1 had only a mild effect on the FI, which was 80% of the control. In soybean there is a large family of over 15 Gm-PR-5 genes with Glyma14g08380 and Glyma17g36680 being the most closely related to the Arabidopsis PR-5 gene, AT1G75040 (Figure 8). The sequences of the proteins encoded by the Arabidopsis and two most closely related soybean genes are highly conserved.

Three well-studied genes involved in SA are AtPAD4, AtEDS1, and AtEDS5. Previously, we demonstrated that the expression of AtPAD4 greatly decreased the development of female SCN to 32% of the control [60]. Here, we examined the effect of overexpression of AtEDS5 and AtEDS1, neither of which significantly affected SCN development, with FI values of 77 and 109, respectively.

SA can be synthesized through a shorter pathway involving ICS, or through a longer pathway through phenylalanine using CM. Therefore, we expressed AtICS1 in soybean roots to determine their effects on SCN maturation. Overexpression of AtICS1 in roots had a modest inhibitory effect (FI = 67, P = 0.002). Because AtICS1 did not strongly affect the FI, we anticipated that expression of CM would have minimal inhibitory effect on the FI of SCN or, perhaps, increase susceptibility, because CM competes with ICS for the common substrate chorismate. However, expression of AtCM-3 also significantly inhibited expression of CM with FI values of 77 and 109, respectively.

WIN3, encoded by HOPW1-1-INTERACTING 3 (WIN3), is involved in regulating SA and disease resistance [70-72], though the mechanism is unclear [73]. Overexpression of AtWIN3 decreased the FI of SCN to 47% of the control.
pathogenesis-related genes PR-1 (unknown function), PR-2 (β-1,3-glucanase), and PR-5 (osmotin), and resistance to P. syringae DC3000 was dependent upon the NPR1 mediated signaling pathway [75]. Overexpression of AtACBP3 in soybean roots resulted in a decrease of the FI of SCN to 53% of the control. Overexpression of AtCPR5 (CONSTITUTIVE EXPRESSOR OF PATHOGEN RELATED GENES 5) in soybean roots had little effect on the female index of SCN. CPR5 mutants constitutively express PR genes at a high level [76,77]; display defects in cell division, endoreduplication, and cell wall production [78,79]; and are of reduced stature and exhibit the formation of spontaneous lesions [79,80].

JA-related genes

JA and related compounds are important in defense responses, especially the response to necrotrophic pathogens [81,82]. JA and JAs are synthesized through a series of

Figure 7 Multiple sequence alignment of the Arabidopsis and soybean protein sequences of TGA2-1B using Clustal 2.1, showing the bZIP domain (bold) and the DOG1 domain (underlined). (*) = identical aa; (: ) = highly conserved aa substitution; (.) = conserved substitution.

Figure 8 Multiple sequence alignment of the Arabidopsis and soybean protein sequences of PR-5 using Clustal 2.1. (*) = identical aa; (:) = highly conserved aa substitution; (.) = conserved substitution.
enzymatic steps (Figure 2), including the enzymes allene oxide synthase (AOS (DDE2); EC 4.2.1.92); allene oxide cyclase (AOC; EC 5.3.99.6); and jasmonic acid-amido synthetase (JAR1; EC 6.3.2.2). JAR1 conjugates JA with isoleucine to form JA-Ile, which is considered to be one of the active forms of JA [74-77]. Overexpression of the Arabidopsis genes AtAOS, AtAOC, and AtJAR1 did not influence the FI of SCN in a statistically significant manner (66% (P = 0.11), 76% (P = 0.06), and 69% (P = 0.07) of the control, respectively; Table 1). These data do not suggest overexpression of these genes will improve resistance in soybean to SCN.

**Other Arabidopsis genes**

Overexpression of AtRIN4 genes in soybean roots had little effect on the female index of SCN (Table 1). RIN4 is a negative regulator of innate immunity in plants [57]. It regulates stomatal closure. It appears to be peripheral to the defense response of soybean roots to nematode attack, as it did not significantly alter the FI of SCN.

In Arabidopsis, the chloroplast protein ACCELERATED CELL DEATH 2 (ACD2) modulates the amount of cell death that occurs in Arabidopsis leaves infected with *P. syringae* [84]. When the AtACD2 gene was overexpressed in soybean roots, the FI of SCN was reduced to 55% of the control (Table 1).

Cysteine endoproteinases containing a C-terminal endoplasmic reticulum retention signal, KDEL, are involved plant cell death [85]. Overexpression of the cysteine endoproteinase encoded by *AtCEP1* reduced the FI of SCN to 66% of the control which was not statistically significant (Table 1).

**Arabidopsis genes that increased susceptibility when overexpressed**

The *AtDND1* gene AT5G15410.1 encodes the cyclic nucleotide-gated cation channel protein DEFENSE NO DEATH 1 (DND1), and is involved in production of the hypersensitive response [86]. The Arabidopsis *dnd1* mutant produces elevated amounts of SA. Overproduction of *AtDND1* in soybean roots did not provide resistance to SCN; rather, it enhanced susceptibility. The FI of transgenic soybean roots containing *AtDND1* was 175% of the control, the largest increase in susceptibility of the genes tested here (Table 1). The protein sequence of DND1 is highly conserved between Arabidopsis and soybean (Glyma18g49890.1) as indicated in Figure 9. It contains a cyclic nucleotide-binding domain as indicated by a significant (e-value = 5.8) Pfam-A match.

Overexpression of two other Arabidopsis genes did not alter susceptibility of soybean to SCN at a statistically significant level. The first gene, CONSTITUTIVE DISEASE RESISTANCE 1 (*AtCDR1*), encodes an aspartic protease [87]. When *AtCDR1* was overexpressed in soybean roots, the FI was 142% of the control (P = 0.23) (Table 1). The second gene *AtMC2* (*LOL2* (*LSD1-LIKE*)) encodes the positive regulator of cell death during the hypersensitive response and is a conserved paralog of LSD1 [88,89]. LSD1 is a negative regulator of plant programmed cell death. Overexpression of *AtMC2* in soybean roots yielded a FI of 135% (P = 0.06) (Table 1).

**Discussion**

Resistance to SCN is a multigenic trait and several genetic loci have been mapped [15,27-29,90-93]. Recently, the identity of genes residing at the *rhl1* and *rhl4* loci have been reported [6-9] which confer some resistance to SCN. However, none of these loci alone provides full resistance to any one SCN population. For example, in a cross between soybean cv Essex and Forrest, *rhl1* and *rhl4* accounted for about 65% of the variation in resistance found in the resultant inbred population to SCN [94]. Other soybean genes have been identified that confer partial resistance to SCN when overexpressed in roots [8,9,60,61].

An option to developing resistance to nematodes is to use defense-related genes that have been described in the literature. Much of the literature describing work with the defense response of Arabidopsis is concerned with elucidating defense response signaling, regulation, and pathways important to bacterial and fungal pathogens that attack the leaf of the plant. Although this research may be applicable to resistance of plants to nematodes and to agronomic crops such as soybean, little published work has yet translated the knowledge gained from these important studies in Arabidopsis to soybean and other important crops. Direct translation of research in Arabidopsis, includes transforming Arabidopsis genes directly into crop plants to determine if they have a positive or negative effect in that crop on disease resistance. Here we have shown that some Arabidopsis genes, when overexpressed in soybean roots, are compatible and confer resistance to SCN.

SA plays an important role in the plant defense response to pathogens. SA regulates SAR, local disease resistance, host cell death, and expression of genes involved in the defense response [44]. In tomato, SA is important to resistance to three RKN species [21]. Transgenic tomato expressing *NahG*, encoding salicylate hydrolase which degrades SA, was less resistant to RKN. However, resistance to RKN induced in tomato through the application of cell suspensions of the biocontrol bacterium *Pseudomonas aeruginosa* is independent of the accumulation of SA [95]. Thus, it may be that SA plays a role in providing resistance to RKN in tomato, but there may be other SA-independent mechanisms that also confer resistance. Uehara et al. [96] showed that inhibition of the SA signaling pathway in tomato harboring the *Hero A* gene increased susceptibility to *Globodera rostochiensis*. A protective effect against gall eelworm was seen in tomatoes when seeds were soaked in SA [14]. These and other reports show a strong link between SA and nematode resistance.
Examination of Arabidopsis mutants has played a key role in our understanding of the defense response and is the subject of many reviews [17,18,35,36,38,39]. It is postulated that SA can be synthesized through two different biochemical pathways [52,97]. In the first pathway, chorismate is converted to isochorismate via the action of ICS; then, SA is produced from isochorismate by isochorismate pyruvate lyase. Examination of ICS1 mutants, sid2-1 and sid2-2, of Arabidopsis indicate that loss of ICS1 activity dramatically decreases SA levels [48]. Most SA synthesized and relevant to plant defense in Arabidopsis appears to be made through this pathway. The sid2 mutant does not accumulate SA upon inoculation with P. syringae, and PR-1 expression is reduced greatly. However, PR-2 and PR-5 are expressed [90]. The second possible pathway diverts chorismate via CM to phenylalanine, which is converted to cinnamic acid by phenylalanine ammonia lyase (PAL) and progresses through a series of reactions to form SA. Previously, we demonstrated that overexpression in transgenic soybean roots of two different soybean genes encoding PAL did not greatly affect SCN maturation, with FI values of 94 and 111% [8]. However, here we show that overexpression of CM and ICS, representatives of the two different pathways, decrease the FI to 57% and 67% of the control, respectively. However, overexpression of PAL, CM, or ICS alone does not confer resistance to the level provided by overexpression of several SA-related genes individually.

Genes decreasing susceptibility of soybean to SCN

If the SA-related defense response is a major factor in soybean resistance to SCN, then components regulated

| AT5G15410.1 | Glyma18g49890.1 |
|-------------|-----------------|
| MPSPNFIFPWR1GLFSDKFRQGQTTIDENEQLNQDSSSSSSDTEPLSLVSRBACTQ | --- | ...
| VQFVPFSTDCD-QAHAPRASAGAGSSSLPIE-EVPMNAPRFRKLRQGFPFRVLDPF | VQFVPFSTDCDGSAGHSLQWEASAGSLPIE-SFPFRKVRKFPQTVSRKQHGRVACTQ |
| KRVQRAWALLGALAMALPDFFYALSQGTKCLYMDAQPVSTCGLAVH | KRVQRAWALLGALAMALPDFFYALSQGTKCLYMDAQPVSTCGLAVH |
| LHNWQLQPRLAFVSRESLVUCCKLVWDREAASHEASLVRESLVUCCKLVWDREAASHE | LHNWQLQPRLAFVSRESLVUCCKLVWDREAASHEASLVRESLVUCCKLVWDREAASHE |
| VVPLRLREEEKVLINTILLILPFGPLFKHYICLMRRKQTVTGAFPIMWFPAPALNI | LVPLRLREEEKVLINTILLILPFGPLFKHYICLMRRKQTVTGAFPIMWFPAPALNI |
| AYFIASHAGCQCVLIAQKAVSAICQMNCTMNLSLCCCVICQYQTPSTTVGFC | AYFIASHAGCQCVLIAQKAVSAICQMNCTMNLSLCCCVICQYQTPSTTVGFC |
| GCN--STVKEKLCLDVEFGKKGQVAPLNIWMEVAIFQ | GCN--STVKEKLCLDVEFGKKGQVAPLNIWMEVAIFQ |
| EPTSNWLEVIFSIVMVLSGLLLFTLLIGNIQVFLHAVMAKRRQICRCDGMWMMRGL | EPTSNWLEVIFSIVMVLSGLLLFTLLIGNIQVFLHAVMAKRRQICRCDGMWMMRGL |
| PSRLQVRKVRQFERQWNALGDECELEIHLPLGRDIKLYKLDLKYYVPFRMDLD | PSRLQVRKVRQFERQWNALGDECELEIHLPLGRDIKLYKLDLKYYVPFRMDLD |
| ILNIDCRKVRKVPKDEIKIREDVPVMFIFMGRVKIRQSLGVLATSTLEGQYL | ILNIDCRKVRKVPKDEIKIREDVPVMFIFMGRVKIRQSLGVLATSTLEGQYL |
| GDELLSWCLRFDFDLPLPSATFVCLENIEAPSQDLERIDYDHRKFYKAFANERLKTA | GDELLSWCLRFDFDLPLPSATFVCLENIEAPSQDLERIDYDHRKFYKAFANERLKTA |
| RYSSNWRWAVAWNQMIFKRRKGFRQGENIGSMPCVSEMSINGEstGERLQAYAFKMS | RYSSNWRWAVAWNQMIFKRRKGFRQGENIGSMPCVSEMSINGEstGERLQAYAFKMS |
| IRPDHDHE | IRPDHDHE |

Figure 9 Multiple sequence alignment of the Arabidopsis and soybean protein sequences of DND1 using Clustal 2.1. (*) = identical aa; (:)= highly conserved aa substitution; (.) = conserved substitution.
by SA may confer resistance to SCN when overexpressed. Our results show that several Arabidopsis genes involved in SA regulation, synthesis, and signaling conferred resistance to SCN when overexpressed in soybean roots. The Arabidopsis genes AtNPR1, AtTGA2, AtPR-5, and several others related to SA strongly decreased the FI of SCN in transgenic soybean roots (Figure 1). NPR1 is a master regulator of the SA-related defense response, and it is a receptor for SA [51]. NPR1 binds SA and interacts with TGA transcription factors, such as the transcription factor AHBP-1b/TGA2, perhaps through its ankyrin domain [54]. NPR1 and TGA transcription factors work downstream of SA and are important to the expression of the genes encoding PR-1, PR-5, and others [98-102]. Expression of these genes is completely abolished in Arabidopsis plants carrying the npr1 mutation [83]. Recently, Pant et al. [103] demonstrated that a Gm ortholog, Glyma09g02430, of Arabidopsis NPR1 reduced SCN cysts to approximately 30% of the control, in agreement with our data for overexpression of AtNPR1 (FI = 33%). There are reports in numerous plants indicating that overexpression of NPR1 results in defense against fungal and bacterial pathogens. Overexpression of AtNPR1 in Arabidopsis conferred resistance to P. syringae and Peronospora parasitica [104]. Overexpression of AtNPR1 in rice conferred resistance to the rice bacterial blight pathogen Xanthomonas oryzae [105]. Overexpression of AtNPR1 in wheat conferred resistance to fusarium head blight, caused by Fusarium graminearum. The apple MpNPR1 gene confers resistance to two fungal pathogens of apple, Venturia inaequalis and Gymnosporangium juniper-virginianae [106,107] complemented Arabidopsis npr1-1 mutants with soybean homologs GmNPR1-1 and NPR1-2, and PR-1 was induced in the transformed plants after infection with P. syringae and after treatment with the SAR inducer, 2,6-dichloroisonicotinic acid.

NPR1 interacts with the transcription factor TGA2 to modulate expression of some plant defense genes, such as PR-1 and PR5 [108]. When we overexpressed AtTGA2, the FI of SCN was decreased to 38, showing that TGA2 can also confer resistance to SCN. This is further supported by our data showing the reduction of the FI to 38% of the control due to PR-5 overexpression. PR-5 is a thiamatin-like protein involved in the defense response [109], perhaps creating transmembrane pores to disrupt the membranes of pathogens [110]. Some PR-5 proteins exhibit anti-fungal activity [109,111]. When Prunus domestica PR-5 was overexpressed in transgenic Arabidopsis, the plants displayed more resistance to the fungal pathogen Alternaria brassicola [112].

PAD4 is found upstream of SA production ([113]; Figure 1). PAD4 is a lipase like protein [48] that can form molecular complexes with EDS1 to modulate SA defense signaling [114]. Ectopic expression of PAD4 reduces feeding time of green peach aphids on transgenic Arabidopsis plants. The aphid spends more time actively feeding on pad4 mutants [115]. Previously, we showed that AtPAD4 conferred resistance to SCN and RKN [60]. EDS1 is also upstream of SA, and it can interact with PAD4 [114,116,117]. Recently, Pant et al. [103] demonstrated that GmEDS1 (Glyma06g1890) had a great effect on SCN and reduced SCN cysts by approximately 80%. This is in contrast to our data indicating that overexpression of AtEDS1 did not decrease the FI of SCN. GmEDS1 is composed of 620 aa and AtEDS1 is composed of 623 aa (Figure 10). The amino acid sequences of GmEDS1 and AtEDS1 have 239 aa in common. Furthermore, they have another 140 aa that are closely related substitutions. Apparently, this conservation is not enough for AtEDS1 to provide resistance to SCN as did GmEDS1.

The EDS5 gene, also found upstream of SA, encodes a membrane protein with homology to multidrug and toxin extrusion (MATE) transporters [50]. The eds5 mutant accumulates very little SA and exhibits a reduction in PR-1 transcripts when infected with nematodes [19,118]. We show that overexpression of AtEDS5 reduced the number of mature female cysts only modestly, to 75% of the control.

ACBP3 is one of six acyl-coenzyme A (CoA)-binding proteins in Arabidopsis [66]. ACBP binds to acyl-CoA esters and protects acyl-CoAs from degradation [119]. Bovine ACBP overexpression in yeast leads to an increase in the acyl-CoA pool size [120]. Overexpression of AtACBP6 increased freezing tolerance in Arabidopsis [121]. These plants also showed a decrease in phosphatidyl choline and an increase in phosphatidic acid. Infection of Arabidopsis by either Botrytis cinerea or P. syringae pv tomato DC3000 induces the expression of AtACBP3, as does treatment with the fungal elicitor arachidonic acid [75]. The authors also showed that resistance to P. syringae was conferred by ACBP3 overexpression in an NPR1-dependent manner and that PR-1, PR-2, and PR-5 were constitutively expressed. When we overexpressed AtACBP in transgenic soybean roots, the number of SCN cysts decreased to 53% of the control at 35 dai.

The ACD2 gene in Arabidopsis encodes red chlorophyll reductase [122], which catalyzes the degradation of the porphyrin portion of chlorophyll [123]. ACD2 modulates cell death in Arabidopsis infected with P. syringae. It is localized to the chloroplast. Upon infection by P. syringae, localization of the protein changes, and it is localized in chloroplasts, mitochondria, and to a lesser degree the cytosol [84]. The accumulation of chlorophyll breakdown products may trigger cell death [122]. When we overexpressed the AtACD2 gene in soybean roots, the FI of SCN was reduced to 55% of the control.
Jasmonic acid

JA and ethylene are also important in the plant defense response, especially the response to necrotrophic pathogens [58]. JA and ethylene interact antagonistically with SA. Mechanical damage and wounding activates JA synthesis in Arabidopsis, potato, tomato, and other plants [58,124,125]. The role of JA in defense against nematodes is being examined by several laboratories. There are recent reports that JA is involved in defense in rice plants against nematodes [126,127]. Exogenous application of methyl-JA on rice shoots reduced galls by 63% per plant. In contrast, Bhattarai et al. [128] showed that JA is not required for resistance in tomato to RKN. They used nearly isogenic tomato cultivars resistant and susceptible to RKN to study gene expression using microarrays. The tomato jai1 mutant, altered in JA signaling, reduced the susceptibility of tomato to RKN. Furthermore, they showed that auxin-related genes were differentially expressed in compatible and incompatible interactions with RKN. Neither foliar spray nor soil-drenching of tomato plants with SA, JA, or methyl-JA affected galling of roots by RKN [129]. We show that overexpression of three genes involved in JA/JA ile production, AtAOS, AtAOC, and AtJAR1 reduced the FI of SCN to 66% (P = 0.06), 75% (P = 0.6), and 69% (P = 0.06) of the control. Although, the data are at the borderline of significance, the trend suggests that JA/JA ile may provide some degree of resistance to SCN in soybean. Further work is needed in this area, as the effects of SA and JA in roots has not been explored. Nor has SA-JA antagonism been documented in root systems. Perhaps, SA and JA interactions are not completely antagonistic at all times in all tissues. Or perhaps, exogenous application of plant hormones...
can provide resistance to nematodes, but the level of the plant hormone necessary to achieve resistance is not normally achieved during nematode attack.

**Genes increasing susceptibility of soybean to SCN**

Overexpression of *AtDND1* resulted in the greatest increase in susceptibility of soybean roots to SCN of all genes tested here. DND1 is a known negative regulator of plant immunity [130-132]. Its promoter is the target of the transcriptional co-repressor, Topless-related 1 (TPR1), which may function through repression of negative regulators to activate R protein-mediated immunity responses [133]. When *AtDND1* was overexpressed in soybean roots, it decreased resistance to SCN as reflected by the female Index of 175% as compared to the control.

**Arabidopsis defense genes not impacting SCN susceptibility**

Overexpression of *AtCDR1*, which encodes an apoplastic aspartic protease, resulted in a non-significant increase in susceptibility to SCN. This result contrasts with those of previous studies on plant resistance to bacterial and fungal pathogens. Overexpression of *CDR1* in T-DNA activation tagging studies yielded dwarf *Arabidopsis* plants and increased resistance to *P. syringae* [134]. Antisense *CDR1* *Arabidopsis* plants were compromised in resistance to *P. syringae*. A rice aspartic protease, encoded by OsCDR1, was identified by Presad et al. [135]. When they overexpressed OsCDR1 in *Arabidopsis*, the plants were more resistant to the necrotrophic fungal pathogen *Alternaria brassicicola*. When the gene was overexpressed in rice plants, the plants were more resistant to *Xanthomonas oryzae*, the rice blast fungus, and to *Magnaporthe oryzae*, which causes bacterial blight.

**Conclusions**

Expression of several *Arabidopsis* genes provided protection to soybean against SCN. In fact, several genes provided increases in resistance comparable to or better than that provided by *Rhg1* and *Rhg4*, two naturally occurring resistance gene loci in soybean. However, not all *Arabidopsis* genes provided resistance. In fact, overexpression of *AtEDSI* did not increase resistance, although a *GmEDS1* ortholog was recently reported as providing resistance. These data indicate that some *Arabidopsis* genes can be used directly in soybean to confer resistance, especially genes associated with SA regulation, signaling, and synthesis, but not all *Arabidopsis* orthologs will provide the same results as the orthogonal soybean gene. These and similar studies may provide useful insights into protein conservation and function, and several of these *Arabidopsis* genes may prove useful in engineering plants with broad resistance to nematodes.

**Methods**

**Bioinformatics**

Thirty-one genes were selected from published studies defining *Arabidopsis* mutants displaying phenotypes affecting SA and JA production, regulation, and signaling. The DNA sequence of the gene was obtained from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/). The DNA sequences of soybean genes used in multiple sequence alignments with *Arabidopsis* genes were obtained at Phytozone.net (Joint Genome Institute, U.S.D.O.E., Center for Integrative Genomics, U.C. Berkeley) using the *Glycine max* genome [136]. Primers for PCR amplification of the open reading frame of each gene were designed using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) or OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, IA.) Multiple sequence alignments were made using CLUSTAL 2.1 (http://www.genome.jp/tools/clustalw/). Protein domains were identified using Pfam (http://pfam.sanger.ac.uk/search).

**Amplification and cloning of ORFs**

The open reading frames (ORFs) of *Arabidopsis* target genes (Additional file 1: Table S1) were amplified by PCR and cloned into pRAP15 using the Gateway® system (Invitrogen, Carlsbad, CA) as described previously [8,60]. Templates for PCR were from cDNA libraries derived from *Arabidopsis* RNA. *Arabidopsis* CDNA was constructed from RNA extracted from *A. thaliana* (Columbia) whole plants and converted into cDNA as described by [8,60]. ORFs were PCR amplified using gene-specific PCR primers that contained CACC at the 5′ end of the forward primer for directional cloning using the Gateway® (Invitrogen) system (Additional file 1: Table S1).

The PCR-amplified ORFs were cloned into pENTR using a pENTR™ Directional TOPO® Cloning Kit (Invitrogen) and transformed into *Escherichia coli* using One Shot™ Mach1™ T-1 chemically competent cells (Invitrogen). Transformed colonies were selected using 50 μg mL⁻¹ kanamycin. Each cloned ORF was DNA sequenced using the vector-specific primers M13-F and M13-R to confirm identity and integrity (Additional file 3: Table S3). Then, each ORF was directionally cloned into pRAP15, a gene expression vector [8,9], at the attR1 and attR2 sites using Invitrogen’s Gateway® technology and LR Clonase™ II Enzyme Mix (Invitrogen). The Clonase II reaction product was used to transform *E. coli* cells, and transformed colonies were selected on 10 μg mL⁻¹ tetracycline plates. Presence of the insert in the correct orientation downstream from the FMV promoter was confirmed by PCR using the FMOV-specific primer FMV-F (Additional file 3: Table S3) and the *A. thaliana* gene-specific reverse primer. The pRAP15 vector bearing each ORF was used to transform competent *Agrobacterium rhizogenes* ‘K599’ cells using the freeze-thaw method [137] with selection on 5 μg mL⁻¹.
tetracycline plates. Presence of the ORF in the pRAP15 vector was confirmed as described above. Expression of the ORF was controlled by the Figwort Mosaic Virus (FMV) promoter. The pRAP15 vector contains the gene encoding enhanced green fluorescent protein gene (eGFP) [138] regulated by the rolD promoter to provide strong eGFP expression in the root for identification of transformed roots. Presence of the gene encoding eGFP was confirmed by PCR using eGFP-F and eGFP-R primers (Additional file 3: Table S3), and eGFP was confirmed visually in transgenic roots. Presence of the A. rhizogenes Rl plasmid was confirmed by PCR using Rl-F and Rl-R primers (Additional file 3: Table S3).

Formation and confirmation of composite soybean plants
Composite soybean plants consisting of untransformed shoots and transformed roots were produced as described previously [139,140] A. rhizogenes clones containing each ORF were grown as described previously [8]. Transformed control roots were produced using A. rhizogenes containing empty pRAP15 with no ORF. Briefly, one hundred soybean cv. Williams 82 PI518671 plants were grown in Promix in the greenhouse for 5–7 days. The plantlets were cut at the soil line and transformed with A. rhizogenes grown to an OD_{600} of 0.5. The stems were rinsed, and the plantlets were planted in the greenhouse and grown for four to five weeks. The plantlets were gently removed from the Promix, and non-transformed roots were excised. Transformed roots were retained after being recognized by fluorescence of eGFP using a Dark Reader Spot lamp (Clare Chemical Research, Dolores, CO). Plants were replanted in Promix and grown an additional two weeks. The non-transformed roots were removed a second time and approximately 12 to 20 healthy plants with only transformed roots were planted in sand and inoculated with SCN.

The presence of Arabidopsis genes in soybean transgenic roots was confirmed by PCR. Briefly, transgenic soybean roots from each construct were harvested, flash-frozen in liquid nitrogen in 2 ml microfuge tubes, and stored at -80°C. After grinding 100 mg root tissue with a mortar and pestle in liquid nitrogen, total RNA was extracted using the RNAeasy Plant Mini Kit (Qiagen, Valencia, CA). Extracted samples of RNA were treated with TURBO™ DNase I (Ambion, Carlsbad, CA) to remove residual genomic DNA. RNA was tested for genomic DNA contamination by PCR amplification using soybean primers for the soybean gene AW31036. No amplification products were produced when the RNA samples were used as template, but an amplification product was produced when genomic DNA served as template. One milligram of each RNA was converted to cDNA using the Superscript III First-Strand Syntheses System for RTPCR (Invitrogen, Carlsbad, CA), using oligo (dT)$_{12-18}$ to prime the first strand of cDNA. To test the presence of the Arabidopsis constructs in the transgenic soybean roots, each cDNA served as template for amplification using gene-specific primers in a PCR reaction with Taq DNA polymerase (Invitrogen). Ten microliters of each reaction was electrophoresed on a 2% SB agarose gel for one hour at 150 volts. A 1Kb Plus ladder (Invitrogen) was included to estimate the size of the amplicons. The gel was photographed using a UV light box with an EOS Rebel T3i camera (Canon, Arlington, VA) with a HD UV filter (Canon). Images created with EOS imaging software (Canon) were annotated in Adobe Imaging software (Adobe, San Jose, CA). The nine gene-specific primers were tested by PCR to confirm that Arabidopsis cDNA was the source of the amplicon and not soybean DNA. PCR containing Arabidopsis cDNA produced amplicons, and only the positive control soybean-derived primer pairs gave amplification products when soybean DNA was used, confirming that the primers were specific to only the Arabidopsis genes within the soybean roots.

Preparation of nematodes
SCN line NL1-RHg was maintained on susceptible Glycine max cv. ‘Essex’ as described previously [141]. Roots were washed to dislodge SCN cysts, which were captured between nested 850-μm and 250-μm sieves. Cysts were purified by sucrose flotation [142] and crushed against a 7.6-cm diameter 250-μm sieve with a rubber stopper partially submerged in water to release the eggs. Eggs captured in a tray below the sieve were poured through a 61-μm sieve and collected on a 25-μm sieve. Eggs were cleaned by soaking in 0.5% sodium hypochlorite for 1.5 minutes, and then rinsed in sterile deionized distilled water. The eggs were poured into a small tray and hatched in a solution of 3 mM ZnSO$_4$ on a rotary shaker at 26°C and 25 rpm. After four days, the hatching solution was passed through a 30-μm mesh nylon cloth (Spectrum Labs Inc, Rancho Dominguez, CA), which retained the unhatched eggs and liberated the J2 stage SCN in the solution collected below the cloth. To concentrate the J2s, 200 mL of the solution was placed in a 1 L glass beaker and placed on a rotary shaker at 100 rpm. J2s were collected from the center bottom of the beaker with a Pasteur pipette. Three 5-μL aliquots of the J2 solution were examined under the microscope to determine the concentration and viability of the J2s. The solution was diluted to a concentration of 1000 J2 mL$^{-1}$ with sterile water.

Nematode assay
Twelve transformed composite plants for each construct tested were inoculated with 2000 J2 nematodes per plant. Two holes 4-cm deep were made in the sand on either side of each plant. One mL of a 1000 J2 mL$^{-1}$ suspension
was added to each hole and covered with sand. At 35 days after inoculation (dai), the cysts were collected from the roots of each plant between nested 850-μm and 250-μm sieves and rinsed onto lined filter paper in a Buchner funnel under vacuum [143]. Cysts were counted under a dissection microscope. Plant roots transformed with empty vector were used as the positive control for the female index as described below.

PCR assays
Expression of ORFs in transformed soybean roots was confirmed by RT-PCR as described previously [60]. Three individual soybean roots were harvested per plant serving as a replicate. Experimental replicates may or may not be performed. All of the experiments presented here were conducted according to published procedures, such as those of Golden et al. [153], Riggs and Schmidt [149,150] Kim et al. [151] and Niblack et al. [152]. In the experiments of Golden et al. [153], the labs that originally developed and modified the FI, the FI is typically calculated from a total of 10 or more control plants or the FI is typically calculated from a total of 10 experimental and 3 control plants, each individual plant serving as a replicate. Experimental replicates may or may not be performed. All of the experiments presented here were conducted according to published procedures, such as those of Golden et al. [153], Riggs and Schmidt [149,150] Kim et al. [151] and Niblack et al. [152].

Synthesis System (Invitrogen, Carlsbad, CA) and oligo dT primes were added to each hole and covered with sand. At 35 days after inoculation (dai), the cysts were collected from the roots of each plant between nested 850-μm and 250-μm sieves and rinsed onto lined filter paper in a Buchner funnel under vacuum [143]. Cysts were counted under a dissection microscope. Plant roots transformed with empty vector were used as the positive control for the female index as described below.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**
Three individual roots (100 mg each) were collected that were transformed with either *AtNPR1*, *AtTGA2*, or the empty pRAP15 vector as control, respectively. Each root represented an independent transformation event. RNA was extracted from each root using a Ultra Clean Plant RNA Isolation Kit (MOBIO, Carlsbad, CA). Genomic DNA was removed using DNase I. Single-stranded cDNA was synthesized from the RNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and oligo dT primers, according to the manufacturer's instructions. All qRT-PCR primer pairs were designed to flank a region that contains one intron to ensure that product was amplified from cDNA. Primers (Additional file 4: Table S4) were specific to the flanking region of the *Arabidopsis* *AtNPR1* and *AtTGA2* genes, yielding amplicons of approximately 150 bp. The soybean ubiquitin-3 (*GmUBI-3*) gene, GenBank accession D28123, served as a positive qRT-PCR control to demonstrate that soybean RNA was present in all samples. Expression levels of the defense-related soybean genes *ERF1* (Glyma20g34570), encoding the ethylene response factor 1; *CHIB1* (Glyma10g27870), encoding a basic chitinase protein; and *PR-5* (Glyma05g38110), encoding an osmotin-like protein also were determined by qRT-PCR. Other controls for qRT-PCR included reactions containing no template and qRT-PCR reactions containing no reverse transcriptase. qRT-PCR was performed on three biological replicates with each reaction replicated three times. The Stratagene Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) was used to determine transcript abundance as described by the manufacturer. SYBR Green was used to measure DNA accumulation during the reaction. The Ct (cycle at which there is the first clearly detectable increases in fluorescence) values were calculated using software supplied with the Stratagene Mx3000P Real-Time PCR system. The dissociation curve of amplified products was used to demonstrate the production of only one product per reaction. To further ensure that only one product was formed in each reaction, the PCR products were analyzed on 0.8% agarose gels and visualized under UV light. Absolute quantification of transcript levels was performed according to the sigmoidal model described by [Rutledge and Stewart, 2008] [62].

**Statistical analysis**
Outliers in the female count data were removed using Grubbs' test [145] at the GraphPad QuickCalc Web site (http://graphpad.com/quickcalc/grubbs1/). Normality of the data was checked using the Shapiro-Wilk test ([146]; online version implemented by S. Dittami, http://scistatcalc.blogspot.com/2013/10/shapiro-wilk-testcalculator.html). Means were compared using Welch's unpaired t test for unequal variance [147,148] at the GraphPad QuickCalc Web site (http://graphpad.com/quickcalc/test1/). The female index (FI) was calculated as follows:

\[ FI = \frac{(N_g/N_c) \times 100}{X} \]

where \( N_g \) = mean number of females for the gene of interest and \( N_c \) = mean number of females for the empty pRAP15 control.

The female index was calculated as described below from 7–16 experimental and 10 or more control plants [8,60,61,139,140,149-153]. Experiments on *Arabidopsis* genes overexpressed in roots of soybean composite plants were conducted according to published procedures, such as those of Golden et al. [153], Riggs and Schmidt [149,150] Kim et al. [151] and Niblack et al. [152]. In the experiments of Golden et al. [153], the labs that originally developed and modified the FI, the FI is typically calculated from a total of 3–10 experimental and 3–10 control plants, each individual plant serving as a replicate. Experimental replicates may or may not be performed. All of the experiments presented
here exceed these published studies in that regard and are conducted with similar plant numbers to recently published studies [8,9,60,61,139,140,154]. In the present analysis, the number of experimental plants met or exceeded that in investigations testing SCN infection in genetically engineered soybean [155-158]. Herein, we also report standard error of the mean (SEM) for experimental and control groups.

Availability of supporting data
The data supporting the results of this article are included within the article.

Additional files

Additional file 1: Table S1. Primers used to PCR amplify ORFs for cloning into pRAP15.

Additional file 2: Table S2. Primers used in RT-PCR assays.

Additional file 3: Table S3. Primers used to confirm clone identity.

Additional file 4: Table S4. Primers used for qRT-PCR.

Abbreviations
AOC: Allene oxide cyclase; ADS: Allene oxide synthase; CM: Chorismate mutase; daii: days after inoculation; ET: Ethylene; F1: Female index; gDNA: genomic DNA; IAA: Indole acetic acid; ICS: Isochorismate mutase; JAs: Jasmonic acid; LOX: Lipoxygenase; OPR3: 12-oxophytodienoic acid reductase; ORFs: Open reading frames; PAL: Phenylalanine ammonia lyase; PAMP: Pathogen-associated molecular patterns; PCR: Polymerase chain reaction; PR: Pathogenesis-related; RNK: Root-knot nematode; SA: Salicylic acid; SAR: Systemic acquired resistance; SCN: Soybean cyst nematode.

Competing interests
The authors have no competing interests.

Authors’ contributions
BM conceived of and designed the experiments, analyzed the data, drafted the manuscript; HB grew the plants, transformed the plant roots, and trimmed the roots; RY cloned the genes, trimmed the roots, harvested and counted the nematodes; SK trimmed the roots, harvested and counted the nematodes, and assisted with experimental design; EB cloned genes, transformed the plant roots, and trimmed the roots; JA: Jasmonic acid; LOX: Lipoxygenase; OPR3: 12-oxophytodienoic acid reductase; ORFs: Open reading frames; PAL: Phenylalanine ammonia lyase; PAMP: Pathogen-associated molecular patterns; PCR: Polymerase chain reaction; PR: Pathogenesis-related; RNK: Root-knot nematode; SA: Salicylic acid; SAR: Systemic acquired resistance; SCN: Soybean cyst nematode.

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Appendix

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