Research article

**Systemic acquired resistance in soybean is regulated by two proteins, Orthologous to Arabidopsis NPR1**

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**Abstract**

**Background:** Systemic acquired resistance (SAR) is induced in non-inoculated leaves following infection with certain pathogenic strains. SAR is effective against many pathogens. Salicylic acid (SA) is a signaling molecule of the SAR pathway. The development of SAR is associated with the induction of pathogenesis related (PR) genes. Arabidopsis non-expressor of PR1 (NPR1) is a regulatory gene of the SA signal pathway [1-3]. SAR in soybean was first reported following infection with *Colletotrichum trancatum* that causes anthracnose disease. We investigated if SAR in soybean is regulated by a pathway, similar to the one characterized in Arabidopsis.

**Results:** Pathogenesis-related gene *GmPR1* is induced following treatment of soybean plants with the SAR inducer, 2,6-dichloroisonicotinic acid (INA) or infection with the oomycete pathogen, *Phytophthora sojae*. In *P. sojae*-infected plants, SAR was induced against the bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*. Soybean *GmNPR1-1* and *GmNPR1-2* genes showed high identities to Arabidopsis *NPR1*. They showed similar expression patterns among the organs, studied in this investigation. *GmNPR1-1* and *GmNPR1-2* are the only soybean homologues of *NPR1* and are located in homoeologous regions. In *GmNPR1-1* and *GmNPR1-2* transformed Arabidopsis *npr1-1* mutant plants, SAR markers: (i) *PR-1* was induced following INA treatment and (ii) *BGL2* following infection with *Pseudomonas syringae* pv. *tomato* (*Pst*), and SAR was induced following *Pst* infection. Of the five cysteine residues, Cys82, Cys150, Cys155, Cys160, and Cys216 involved in oligomer-monomer transition in Arabidopsis NPR1, Cys216 in *GmNPR1-1* and *GmNPR1-2* proteins was substituted to Ser and Leu, respectively.

**Conclusion:** Complementation analyses in Arabidopsis *npr1-1* mutants revealed that homoeologous *GmNPR1-1* and *GmNPR1-2* genes are orthologous to Arabidopsis NPR1. Therefore, SAR pathway in soybean is most likely regulated by *GmNPR1* genes. Substitution of Cys216 residue, essential for oligomer-monomer transition of Arabidopsis NPR1, with Ser and Leu residues in *GmNPR1-1* and *GmNPR1-2*, respectively, suggested that there may be differences between the regulatory mechanisms of *GmNPR1* and Arabidopsis NPR proteins.

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Background

Plants use a series of physical, preformed chemical and inducible defense mechanisms to protect themselves from pathogen attack. One of the most common inducible defense mechanisms is systemic acquired resistance (SAR). SAR can be triggered by infection with certain pathogenic strains. The induced resistance is typically effective against a wide range of pathogens including those taxonomically unrelated to the SAR inducing organism [4].

Salicylic acid (SA) is a signaling molecule of the SAR pathway [2,5]. Exogenous application of SA increases the resistance of tobacco plants to tobacco mosaic virus (TMV) [6]. SAR can be induced effectively by exogenous applications of either SA or synthetic functional analogs of SA, 2,6-dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7-carbo-thioic acid S-methyl ester (BTH) [5,7]. In addition to signaling SAR, SA regulates both basal and R-gene mediated local disease resistance mechanisms [8].

The development of SAR is associated with the induction of pathogenesis related (PR) gene expression [6]. Increases in the endogenous SA levels in the pathogen-inoculated plants coincide with the increased levels of the PR gene expression and enhanced disease resistance [9,10]. Transgenic plants expressing the bacterial salicylate hydroxylase (nahG) gene cannot accumulate SA and fail to express SAR development [2,11]. The PR genes, known as the SAR markers, have been identified from several plant species including tobacco and Arabidopsis [4]. A soybean PR1 homolog, GmPR1 is induced by both SA treatment and infection of soybean leaves with soybean mosaic virus (SMV) [12].

non-expressor of PR1 (NPR1) is a regulatory gene of the SA signal pathway [1-3]. NPR1 is also known as non-inducible immunity 1 (NIM1) [3] or salicylic acid insensitive 1 (SAI1) [13]. The NPR1 gene encodes a protein containing a bipartite nuclear localization sequence and two protein-protein interactive domains, a multiple ankyrin repeat domain and a BTB/POZ domain [14-16]. Both motifs mediate the interactions of NPR1/NIM1 protein with other proteins. NPR1 is an oligomeric, cytosolic protein. Either following pathogenic infection or in response to SA treatment, NPR1 oligomer becomes monomer and moves into the nucleus to activate transcription of pathogenesis-related (PR) genes [17]. The NPR1 protein is also homologous to the Iκ-B and the cactus regulatory proteins found in vertebrates and flies, respectively [3,18]. Both genes are involved in pathways controlling innate immunity in animals. The npr1 mutants with mutations in NPR1 are sensitive to SA toxicity. In the npr1 mutant plants, induction of PR genes and pathogen resistance by SA are abolished. In spite of their ability to accumulate SA, mutant plants are unable to induce SAR indicating that NPR1 is required for the SAR signal transduction pathway [14].

SAR inducers have been used in various field studies on several crop plants to reduce disease incidence [19]. In all of these studies, SAR inducers led to reduced disease symptom development. Overexpression of Arabidopsis NPR1 or its orthologues in transgenic plants has been shown to induce broad-spectrum resistance. For example, overexpression of NPR1 led to development of constitutive enhanced resistance against the bacterial pathogen Pseudomonas syringae and the oomycete pathogen Hyaloperonospora parasitica in Arabidopsis [20]. Overexpression of NPR1 and the rice homolog of NPR1, NH1 resulted in enhanced resistance against the blast pathogen, Xanthomonas oryzae pv. oryzae in transgenic rice [21,22]. In tomato, overexpression of the Arabidopsis NPR1 gene resulted in an enhanced level of resistance to bacterial and Fusarium wilts and a moderate level of resistance against gray leaf spot and bacterial spot diseases [23]. Similarly, wheat plants transformed with Arabidopsis NPR1 resulted in enhanced resistance against Fusarium graminearum that causes Fusarium head blight in wheat and barley [24]. These studies suggest that manipulated expression of NPR1 or its orthologues can create broad-spectrum resistance in crop plants, and therefore, could be a suitable strategy in improving crop plants for disease resistance [25].

In the United States, soybean suffers annual yield losses valued at more than 2.6 billion dollars from various pathogenic diseases [26]. SAR in soybean was first reported following infection with Colletotrichum truncatum that causes anthracnose disease [27]. A significant reduction in lesion sizes following C. truncatum infection was noted in epically, when cotyledons were pre-injected with C. truncatum and C. lagenarium spore suspensions [27]. We investigated if SAR in soybean is regulated by a pathway, similar to the one characterized in Arabidopsis. We have shown that there are two orthologous NPR1 copies in soybean. Non-conservation of the Arabidopsis Cys216 residue in GmNPR1s suggests that either conserved Cys82, Cys150, Cys155, Cys160 residues are sufficient for GmNPR1s’ monomerization or some other soybean cysteine residue(s) complements the Arabidopsis Cys216 function.

Results

INA induces the PR-1 gene expression in soybean

Earlier a soybean PR1 homolog, GmPR1 was shown to be induced by both SA treatment and infection of soybean leaves with SMV [12]. It has not been shown if SA can sys-
temically trigger the expression of GmPR1. We determined if GmPR1 is systemically induced in leaves following feeding of soybean roots with INA, a functional analog of SA.

We used INA, a functional analog of SA, to induce GmPR1. We investigated the time course accumulation of GmPR1 transcripts in response to INA treatment and the data are presented in Figure 1. Northern blot analysis of 3-week old INA treated soybean seedlings showed that GmPR1 transcripts were detected as early as 36 h following INA treatment; and thereafter, GmPR1 expression levels continued to increase during rest of the time course. These results confirmed earlier observation of SA-mediated GmPR1 expression in soybean leaves [12].

**Induction of PR-1 gene expression in systemic soybean leaves following Phytophthora sojae infection**

Although it was demonstrated earlier that GmPR1, a SAR marker, was induced in response to SMV infection, it has not been shown if GmPR1 is systemically induced in non-inoculated systemic leaves [12]. For SAR, induction of GmPR1 gene, a SAR marker, is needed in non-inoculated systemic tissue to provide resistance against secondary infection. To determine if pathogenic infection can also lead to GmPR1 expression in systemic tissues, hypocotyls of young soybean seedlings were inoculated with an avirulent *P. sojae* race and GmPR1 expression was monitored at the site of infection and in non-inoculated systemic leaves. Induction of GmPR1 at infection sites was observed as early as on day 1 with a peak on day 2 post inoculation; and thereafter, induction continued until day 9 following inoculation (Figure 2). In the systemic leaves, induction of GmPR1 was clearly observed by day 9 following inoculation (Figure 2). No systemic induction of GmPR1 was observed when only agar medium with no *P. sojae* mycelia was used to inoculate the wounded hypocotyls (data not shown). These results suggested that SAR pathway is active in soybean.

**Induction of SAR following Phytophthora sojae infection**

Field studies suggested that SAR was induced following infection of soybean with certain pathogens [27]. Based on the results presented in Figure 2, we designed an experiment to investigate the extent of SAR induction in soybean. Wounded hypocotyls of 7-day old seedlings were inoculated with avirulent strain of *P. sojae* and subsequently at 9, 13, 17 and 21 days after the inoculation leaves were infected with a virulent bacterial pathogen, *P. syringae* pv. glycinea (*Psg*). Four days following *Psg* inoculation colony forming units (cfu) of the pathogen in infected leaves were determined. Bacterial counts were comparable to that in agar control when leaves were inoculated with the bacterium nine days following *P. sojae* inoculation (Figure 3). Bacterial counts were, 4.9, 2.2 and 2.3 times lower than the agar-controls when leaves were inoculated with *Psg* 13, 17 and 21 days following *P. sojae* infection. However, only at 13 day the difference was statistically significant (Figure 3). These observations suggested that SAR was induced in non *P. sojae* inoculated soybean leaves following hypersensitive response [28] caused by an avirulent *P. sojae* race.

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**Figure 1**

Induction of the soybean PR-1 (GmPR1) gene by INA.

Transcripts levels in three-week old soybean seedlings are shown at various hours following feeding with either 0.5 mM INA or water through the roots. Two young trifoliate leaves per plant were harvested at the indicated time points for RNA isolation. For 0 h treatment, the leaves were harvested just before INA treatments. RNA gel blot analysis was performed using the GmPR1 gene as the probe. h, hour.

**Figure 2**

Induction of GmPR1 following infection of hypocotyls with Phytophthora sojae. Hypocotyls of 8-day old Williams 82 seedlings were inoculated with *P. sojae* race 4 (avirulent strain). The unifoliate and trifoliate leaves and infected hypocotyl tissues were collected for RNA preparations. Northern analysis was performed using GmPR1 as the probe. For 0 day treatment, the leaf and stem tissues were harvested just before inoculation. d, day.
Soybean genome contains two copies of NPR1-like sequences

As a first step towards investigating the molecular components of the SAR pathway in soybean, we determined if the soybean genome contains the orthologue of SAR regulatory gene, Arabidopsis NPR1. A 1.7 kb fragment of a candidate soybean NPR1 homolog was PCR-amplified from the soybean genomic DNA and named GmNPR1. DNA gel blot analysis using the GmNPR1 probe revealed that there are two copies of NPR1-like sequences in the soybean genome (Figure 4). Screening of a soybean bacterial artificial chromosome (BAC) library [29] for GmNPR1-like sequences resulted in identification of 18 BAC clones. DNA fingerprints of these clones for six restriction endonucleases allowed us to group these clones into two classes, Class I and Class II. None of the BAC clones contained both classes of NPR1-like sequences suggesting that they are unlikely tandem genes. Screening of a soybean cDNA library prepared from etiolated hypocotyls with GmNPR1 resulted in identification of 19 putative clones. These clones were also grouped into two classes based on their restriction patterns. One near full-length cDNA clone for each GmNPR1-like sequence was sequenced. We named these two NPR1-like sequences, GmNPR1-1 (Accession No. FJ418595) and GmNPR1-2 (Accession No. FJ418597). GmNPR1-1 and GmNPR1-2 cDNAs share 96% amino acid identity. Both GmNPR1-1 and GmNPR1-2 shared 40% amino acid identity with Arabidopsis NPR1 (AAC49611) (Figure 5). The cDNA sequences were identical with their corresponding genomic sequences obtained from plasmids p143K5Xb1-2.1 (GmNPR1-1) (Accession No. FJ418594) and p101F23E1-2 (GmNPR1-2) (Accession No. FJ418596). Data obtained from DNA blot analysis and

Figure 3
SAR induction following Phytophthora sojae (avirulent) infection in soybean. Colony forming units (cfu) of P. syringae pv. glycinea (Psg) per leaf in the samples inoculated with Psg 9, 13, 17 and 21 days following exposure of wounded hypocotyls to agar pieces containing either no P. sojae mycelia (solid gray) or P. sojae mycelia (solid black) are shown. Ten microliter droplets of either bacterial cell suspensions (10^7 cells/ml) or 10 mM magnesium chloride were used to inoculate the youngest trifoliate. The study was conducted with three biological replications. Bars without a common letter on the top are statistically different (Fisher’s LSD test, P = 0.05). Standard errors are represented by error bars.

Figure 4
Genomic organization of GmNPR1. Genomic DNA prepared from leaves of the cultivar Williams 82 and digested with four restriction enzymes suggested that there are two copies of GmNPR1 in the soybean genome.
Comparison of GmNPR1-1 and GmNPR1-2 sequences with that of Arabidopsis NPR1

**Figure 5**
Comparison of GmNPR1-1 and GmNPR1-2 sequences with that of Arabidopsis NPR1. Broad-complex Tramtrack Bric-a-brac/Poxvirus and Zinc finger domain (BOB/POZ) is represented by bold letters and Ankyrin repeat domain (ANK) is underlined. Five Arabidopsis cysteine residues (Cys85, Cys159, Cys166, Cys169 and Cys216) regulating NPR1 functions are marked with rectangular boxes. "*" represents identical residues; ":" means conserved substitutions between similar residues; "." indicates the semi-conserved substitutions between similar residues.
characterization of BAC and cDNA clones strongly indicated that the diploidized tetraploid soybean contained two NPR1-like sequences. In order to confirm this conclusion, we conducted nucleotide sequence comparison of the GmNPR1 genes with the soybean genome sequence http://www.phytozome.net/search.php?show=blast.

GmNPR1 genes were identified in two scaffolds (scaffolds_159 and _213) of the soybean genome sequence. GmNPR1-1 is located in Scaffold_159 and GmNPR1-2 in Scaffold_213. Flanking regions of the two genes were compared for possible microcolinearity. High conservation of gene sequences between the two genomic regions suggested that the two GmNPR1 genes are homoeologous and were evolved during the polyploidization event (Additional File 1).

We investigated if there were any additional GmNPR1-like sequences in the soybean genome. We conducted a search for similar soybean EST sequences using tblastx program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This led to identification of a GmNPR1-1-like sequence (BE801977.1) with 58% amino acid identity to GmNPR1-1. Duplicated copies of this sequence, GmNPR1-1-like-1 and GmNPR1-1-like-2, were identified from Scaffolds_15 and _90 of the soybean genome sequence http://www.phytozome.net/search.php?show=blast. These two genes are located in homoeologous regions suggesting that they were also duplicated during polyploidization event (Additional File 2). No significant nucleic acid identity of these two GmNPR1-1-like sequences to either of the GmNPR1 genes was observed. Proteins encoded by these two homoeologous genes are truncated and do not contain more than 110 residues of the N-terminal core BTB/POZ domain required for SA-mediated activation of NPR1 (Additional File 3; [30]). Thus, most unlikely they are involved in SAR pathway.

**GmNPR1 genes are constitutively expressed in soybean**

To study the expression patterns of GmNPR1 genes, RT-PCR analyses were conducted using gene-specific primers on young and old leaves, stems, flowers, young pods, and roots. Presence of an intron distinguished the PCR products of contaminating genomic DNA from that of the reverse transcribed (RT) cDNA templates for GmNPR1 genes. GmNPR1-1 and GmNPR1-2 were constitutively expressed in all soybean organs investigated (Figure 6). RT-PCR analyses of both genes were conducted using the same RT-templates. Therefore, patterns of steady state transcript levels of both genes in various organs were comparable (Figure 6).

**GmNPR1 genes complemented the Arabidopsis npr1-1 mutant**

GS_143K5 and GS_101F23 were selected from Class I and Class II BAC clones, respectively. To investigate if GmNPR1 genes were orthologous to Arabidopsis NPR1, GmNPR1-1 and GmNPR1-2, isolated from these two BAC clones, were transformed into the Arabidopsis npr1-1 mutant carrying the BGL2-GUS fusion gene. Transformants were analyzed to confirm the integration of GmNPR1 genes into npr1-1 by conducting DNA blot analyses. The npr1-1 mutant does not induce PR-1 transcripts following the SA treatment because it lacks NPR1 function. We investigated if GmNPR1 genes, under the control of their native promoters, complemented the npr1-1 mutant and mediated the expression of SAR marker gene, PR-1 in response to INA treatment. Transgenic Arabidopsis npr1-1 mutant plants transformed with either GmNPR1-1 or GmNPR1-2 showed induction of the Arabidopsis PR-1 gene following treatment with INA (Figure 7A). No PR-1 transcripts were detected in water controls (Figure 7). These results suggested that GmNPR1-1 and GmNPR1-2 encode functional NPR1 proteins that were presumably monomerized by INA treatment. The monomeric GmNPR1s then migrated into nuclei and activated transcription of the PR-1 gene. In absence of INA, none of the transgenic plants showed any detectable levels of PR-1 transcripts. These data suggested that cytosolic GmNPR1 migrated into nucleus following INA treatment [17].

The SAR marker BGL2 encoding β-glucanase also requires NPR1 for its induction. The BGL2-GUS fusion gene is silent in npr1-1 because of the absence of NPR1 function...
Figure 7

**Induction of the pathogenesis-related genes by INA or infection in Arabidopsis npr1-1 mutant carrying GmNPR1 genes.** A), Induction of PR-1 gene by INA. RNA gel blot analysis was performed using the Arabidopsis PR-1 gene as the probe. GmNPR1-1: two independent transformants; GmNPR1-2, four independent transformants. Note that PR-1 is induced in GmNPR1-1 and GmNPR1-2 complemented npr1-1 plants. B), Induction of beta glucanase 2 (BGL2) following infection. The leaves of the Arabidopsis npr1-1 mutant carrying the BGL2-GUS fusion gene with the BGL2 promoter transformed with no GmNPR1 gene (a and d), GmNPR1-1 (b and e), or GmNPR1-2 (c and f) were inoculated with Pst just before bolting. a, b, and c were inoculated with a virulent Pst strain. d, e, and f, were inoculated with an avirulent Pst strain. The plants were infiltrated with either Pst DC3000 or Pst DC3000 carrying the AvrRpt2 gene (10^5 cfu/mL (OD<sub>600</sub> = 0.002). Results were comparable in three independent experiments.
To determine if GmNPR1 genes can complement this lost NPR1 function and initiate pathogen-induced BGL2 expression, a transgenic npr1-1 mutant plant carrying either GmNPR1-1 or GmNPR1-2 was tested for expression of GUS driven by the BGL2 promoter. Transgenic npr1-1 plants carrying either GmNPR1-1 or GmNPR1-2 were able to show GUS expression when infected with the avirulent Pst strain containing avrRpt2. These data suggested that both GmNPR1 proteins were able to complement the lost NPR1 function in the npr1-1 mutant and induced pathogen-mediated BGL2 expression (Figure 7B: e, f). No GUS expression was observed in response to a virulent strain, Pst DC3000 carrying no Avr genes (Figure 7B: b, c). BGL2 expression was observed in the distant healthy tissues of the infected leaves (Figure 7B: e, f). Because of cell death, no GUS expression was detected at the infection sites.

Results obtained from three independent experiments strongly suggested that NPR1 function is complemented by both soybean GmNPR1 genes in the npr1-1 mutant.

Discussion

SAR pathway is conserved in soybean

Soybean suffers estimated annual yield loss valued at 2.6 billion dollars from attack of various pathogens [26]. Broad-spectrum SAR has the potentiality to reduce the crop losses from diverse pathogens in soybean. Here we have presented molecular evidence suggesting that the SAR pathway is conserved in soybean. We have isolated soybean genes encoding the SAR regulatory protein, NPR1. Results from Southern blot analysis, gene cloning experiments and soybean genome analyses strongly suggested that there are two NPR1-like sequences in soybean.

We have also shown that in soybean, SAR marker GmPR1 is induced in response to both (i) SAR inducer, INA and (ii) P. sojae infection (Figures 1 and 2).

In soybean, SAR activity against Psg was induced after two weeks of P. sojae infection (Figure 3). However, SAR responses in soybean were not as effective as in some other plant species, such as Arabidopsis thaliana, at least in response to the pathogenic infection tested in this investigation [14]. By three weeks following P. sojae infection, age-related resistance was expressed in both agar-controls and P. sojae-infected seedlings (Figure 3). Age-related resistance has been reported to express in soybean against P. sojae [31,32]. Accumulation of SA but not NPR1 is required for this age-related resistance [33].

Soybean is a diploidalized tetraploid species. Most likely the two GmNPR1 genes were originated from duplication of a single progenitor gene during the polyploidization event. GmNPR1-1 and GmNPR1-2 with 96% amino acid identity are located in two highly collinear homoeologous chromosomal regions (Additional File 1). RT-PCR data suggested that following duplication, promoter activities
of the two genes have been conserved at least for the organs investigated in this study (Figure 6). Both GmNPR1 proteins complemented the lost NPR1 function of the Arabidopsis npr1-1 mutant and mediated the expression of PR-1 and BGL2 following INA treatment and infection, respectively (Figure 7). Further, GmNPR1-complemented npr1-1 plants were able to show induction of SAR following infection with an avirulent pathogenic strain (Figure 8). From these results we conclude that both GmNPR1 genes are orthologous to Arabidopsis NPR1.

**Differences in structure-functional regulations of GmNPR1 and Arabidopsis NPR proteins**

Arabidopsis NPR1 protein interacts with TGA transcription factors in the nucleus to activate the expression of PR1 [34]. Transportation of the NPR1 protein into nucleus is stimulated by SAR inducer [16]. The Arabidopsis npr1-1 mutant carrying either the GmNPR1-1 or GmNPR1-2 showed to initiate PR-1 gene expression following treatment with INA (Figure 7). No PR-1 induction was observed in the control INA treated mutant npr1-1 plant or in the water treated npr1-1 plants complemented with either GmNPR1-1 or GmNPR1-2 (Figure 7). In soybean, INA or infection induced accumulation of GmPR1 transcripts (Figures 1 and 2).

In healthy tissues, NPR1 is an oligomeric, cytosolic protein. Following SA treatment, Arabidopsis NPR1 dimers become monomers and move into nuclei to interact with TGA transcription factors for transcriptional activation of PR1 [34]. In previous studies it has been shown that Cys82, Cys150, Cys155, and Cys160 are involved in oligomer-monomer transition [17,35]. First four of these 5 cysteine residues that are present in BTB/POZ domain of NPR1 are conserved in GmNPR1-1 and GmNPR1-2 (Figure 5). Only Cys216 was not conserved. We used the Cys216 containing region of the GmNPR1-1 gene to isolate all available soybean expressed sequence tags and also soybean genome sequence by conducting tBLASTX searches. None of the soybean sequences showed to contain the Arabidopsis Cys216 residue. In this search, we however identified GmNPR1-1-like-1 and GmNPR1-1-like-2 genes that are located in two homoeologous chromosomal regions (Additional File 2). Proteins encoded by the two GmNPR1-1-like genes most unlikely activate the SAR pathway because they are truncated at the N-terminus and do not contain the core BTB/POZ domain required for SA-mediated activation of PR1 (Additional File 3; [30]).

In GmNPR1-1 and GmNPR1-2 transformed npr1-1 plants (i) SAR markers PR1 and BGL2 are induced following INA treatment and infection, respectively and (ii) SAR following infection (Figures 7 and 8). None of the complemented npr1-1 mutant plants showed any detectible levels of PR1 transcripts prior to INA treatment (Figure 7). These results suggested that GmNPR1 proteins become monomers only following infection or treatment with INA. Thus, either Cys82, Cys150, Cys155 and Cys160 were sufficient for GmNPR1 oligomerization, or additional cysteine residue(s) may co-operate with Cys82, Cys150, Cys155, and Cys160 for oligomerization of GmNPR1s in soybean or in the GmNPR1 complemented npr1-1 plants.

In a recent study, S-nitrosylation of Cys156 is shown to play important role in oligomerization of NPR1 in Arabidopsis [35]. In a mutation experiment, where Cys156 was mutated to Asp156, the efficiency of oligomer formation was reduced as compared to the wild type protein [35]. In GmNPR1 proteins, although Cys156 was mutated to alanine, both GmNPR1 proteins complemented NPR1 function in the npr1-1 mutant (Figure 5). Further investigation is warranted to determine the involvement of other Cystein residues in S-nitrosylation in the absence of Cys156.

**Enhancing SAR in soybean**

We have shown that SAR marker, GmPR1 is expressed in response to both INA treatment and P. sojae infection in soybean, and soybean NPR1 orthologues are functional. In soybean, it has recently been demonstrated that RAR1 and SGT1 are required for SAR and are functional [36]. Together, these data strongly suggest that SAR is induced in soybean. Therefore, overexpression of GmNPR1 genes will most likely enhance broad-spectrum resistance in soybean.

**Conclusion**

Complementation analyses in the Arabidopsis npr1-1 mutant suggested that homoeologous GmNPR1-1 and GmNPR1-2 genes are orthologous to Arabidopsis NPR1. Therefore, SAR pathway in soybean is most likely regulated by GmNPR1 genes. Substitution of essential Cys216 residue for oligomer-monomer transition of Arabidopsis NPR1 with Ser and Leu residues in GmNPR1-1 and GmNPR1-2, respectively suggested that there may be differences between the regulatory mechanisms of GmNPR1 and Arabidopsis NPR proteins. Soybean plants showed expression of the SAR marker PR1 gene and SAR following infection, and carry functional GmNPR1 genes suggesting that overexpression of GmNPR1s in transgenic soybean plants may enhance resistance against many pathogens.

**Methods**

**SAR assay following Phytophthora sojae infection**

The green hypocotyls of 7-day-old light-grown soybean cultivar Williams 82 seedlings were slit open for a length of 1.0 cm and P. sojae race 4 mycelia grown in 1/4th strength V8 agar medium were inserted into these wounds [37]. In controls, only agar medium was used to inoculate the wounded hypocotyls. P. sojae race 4 is avirulent to Wil-
BAC clones containing unique sequences were probed with the 32P-radiolabeled EcoRI, XhoI, and PstI DNA fragments of two individual plasmids containing GmNPR1-1 and GmNPR1-2 genes, respectively, under the regulation of their respective native promoters, were selected for further investigation.

**Sequencing of the GmNPR1-1 and GmNPR1-2 genes**

Inserts of p143K5Xb1-2.1 and p101F23E1-2 plasmids containing GmNPR1-1 and GmNPR1-2, respectively, were sequenced by sub-cloning restriction fragments in the pBluescript II KS (+) vector in *E. coli* DH10Ba. Sequencing was accomplished at the DNA Facility, Iowa State University. Sequence contigs were constructed using ContigExpress™ of the Vector NTI Suite program (InforMax Inc., Bethesda, MD). A primer walking approach was applied in filling the gaps of sequence contigs. GmNPR1-1, GmNPR1-2 and Arabidopsis NPR1 (AAC49611) were compared using ClustalW program (European Bioinformatics Institute). Protein domains were identified by searching the conserved domain database (ps blast).

**Isolation of soybean GmNPR1 cDNAs**

A soybean cDNA library was constructed using the pBluescript II XR cDNA library construction kit (Stratagene, La Jolla, CA). Poly(A+) RNAs for the cDNA library were prepared from *P. sojae*-infected hypocotyl tissues of Williams 82 by using the polyAtract mRNA isolation system III (Promega, Inc., Madison, WI). The library was constructed in *E. coli* DH10B α⁺ strains using the pBluecript II XR cDNA library construction kit (Stratagene, La Jolla, CA). Poly(A) RNAs for the cDNA library were prepared from Williams 82 cell suspensions (10⁷ cells/ml) or 10 mM magnesium chloride [39]. Ten microliter droplets of either bacterial cell suspensions (10⁷ cells/ml) were prepared from 2-day old cell cultures grown in King’s B liquid medium [38]. To facilitate bacterial infection, a pricking inoculation technique was used [40]. DNA was extracted from leaves of the soybean cultivar Williams 82. Leaves were inoculated with the bacterial pathogen, *Psuedomonas syringae* pv. glycinea (Psg), at 9, 13, 17 and 21 days after the inoculation with *P. sojae* race 4 mycelia or agar-with no mycelia. Psg cell suspensions (10⁷ cells/ml) were prepared from 2-day old cell cultures grown in King’s B liquid medium [38]. DNA gel blot analysis was conducted as described previously [42]. DNA gel blot analysis was performed with three biological replications. ANOVA was used to compare different treatments. To determine which of the eight treatments differ from each other, Fisher’s least significant difference (LSD) comparisons were performed at P value of 0.05.

**PCR amplification and screening of a soybean BAC library**

A soybean EST (Gm-c1004-4231) showing high identity to Arabidopsis NPR1 was used to develop a primer pair (forward primer: 5'-GAG CCT TCC ATT ATA GTA TCC CTA CIT AC-3'; reverse primer: 5'-GAC CAG CAA ACT CAC ATG ATA GTA TCC CTA CIT AC-3'). The soybean NPR1-like sequence, GmNPR1 was amplified from Williams 82 genomic DNA by conducting PCR at initial DNA denaturation temperature 94°C for 2 min followed by five cycles of 94°C for 30 sec, 65°C for 30 sec with an increment of -1°C per cycle, 72°C for 1 min; then thirty-five cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by a 10 min DNA extension at 72°C. The amplified products were sequenced to confirm the identity of GmNPR1 and used as a probe to screen a soybean Williams 82 BAC library and conduct DNA blot analyses [29].

**DNA gel blot analysis**

DNA gel blot analysis was conducted as described previously [40]. DNA was extracted from leaves of the soybean cultivar Williams 82. DNA was digested with four restriction enzymes (BclI, EcoRI, HindIII, and PstI). Membranes were probed with the 32P-radiolabeled GmNPR1 sequence [41].

**Cloning GmNPR1 genes into the binary vector, pTF101.1**

EcoRI, SrtI, and XbaI DNA fragments of two individual BAC clones containing unique GmNPR1 sequences were cloned into the binary vector, pTF101.1 in *E. coli* DH108a and colonies were screened for DNA fragments containing GmNPR1 genes [42]. Resultant plasmids, p143K5Xb1-2.1 and p101F23E1-2 containing GmNPR1-1 and GmNPR1-2 genes, respectively, were sequenced by sub-cloning restriction fragments in the pBluescript II KS (+) vector in *E. coli* DH108a. Sequencing was accomplished at the DNA Facility, Iowa State University. Sequence contigs were constructed using ContigExpress™ of the Vector NTI Suite program (InforMax Inc., Bethesda, MD). A primer walking approach was applied in filling the gaps of sequence contigs. GmNPR1-1, GmNPR1-2 and Arabidopsis NPR1 (AAC49611) were compared using ClustalW program (European Bioinformatics Institute). Protein domains were identified by searching the conserved domain database (ps blast).

**GmNPR1 expressions in soybean organs**

Leaf, stem, flower, young pod, and root tissues were collected from Williams 82. Leaf, stem, and root tissues were harvested from three-week old plants. Tissues were frozen quickly in liquid nitrogen and stored at -80°C until their use for RNA isolation. Total RNA was isolated from individual samples using the Qiagen RNAeasy Plant Mini kit (Qiagen, Valencia, CA). RNA concentration was determined using a Unico UV-2000 spectrophotometer (Unico, Inc., Dayton, NJ). Gene-specific primers were designed for RT-PCR analyses (GmNPR1-1_Foward: GATGCTGACCTTGTTGTCGAGGGAATTC, GmNPR1-
Transformation of GmNPR1-1 and GmNPR1-2 into the Arabidopsis npr1-1 mutant

Seeds of Arabidopsis npr1-1 genotype were obtained from Arabidopsis Biological Resource Center, Ohio State University. Seeds were grown in Sunshine mix SB3000 universal soils (Sun Grow Horticulture Inc., Bellevue, WA) under continuous fluorescent light. Plants were fertilized weekly with the Miracle-Gro Excel water-soluble fertilizer 15-5-15 (Scotts, Marysville, OH). Plasmids p143K5Xb1-2.1 and p101F23E1-2 containing GmNPR1-1 and GmNPR1-2 genes, respectively, in the binary vector pTiF101.1 were transformed into Agrobacterium tumefaciens EHA101 by electroporation using a Cell-Porator Pulser (Life Technologies, Rockville, MD). A touchdown program (LSD) was used to conduct an oligo-dT primer (TTTTTTTTTTTTTTT) and M-MLV reverse transcriptase (Life Technologies, Rockville, MD) was used as a DNA marker. The gels were photographed with an AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA).

Complementation analysis in transgenic Arabidopsis plants

Ecotype Columbia, npr1-1 mutant, and npr1-1 mutant transformed with either GmNPR1-1 or GmNPR1-2 were selected for investigating the complementation of NPR1 function for SAR activity in the npr1-1 mutant background. Arabidopsis plants were sown in Sunshine LC1 mix (Sun Grow Horticulture Inc., Bellevue, WA) under 9 h light and 15 h dark regimen at 22°C with 55% humidity. After two weeks, seedlings were transplanted. Four weeks following planting fully developed two leaves (leaf number 3 and 4) were inoculated with 10 mM MgCl₂ or an avirulent strain Pst DC3000 containing AvrRpt2. Leaves were inoculated with a syringe containing bacterial cells grown for 48 h in NYG medium containing rifampicin (50 µg/ml) and kanamycin (25 µg/ml) [44]. Bacterial cells for inoculation were collected by centrifugation and then resuspended in 10 mM MgCl₂ to an optical density 0.2 at A₆₀₀ which corresponds to ~10⁸ cfu/ml. Bacterial suspensions were diluted to 10⁷ cfu/ml in 10 mM MgCl₂ [45]. Two leaves per plant were infiltrated with this bacterial suspension using a 1-ml syringe. About 40 µl bacterial suspension (10⁷ cfu/ml) was infiltrated in each leaf. Three days after inoculation, two younger systemic leaves (leaf number 5 and 6) were inoculated with the virulent strain Pst DC3000. Pst DC3000 contains empty vector with the kanamycin resistance gene. Bacterial cells for inoculation were collected by centrifugation and then resuspended in 10 mM MgCl₂ to an optical density 0.001 at A₆₀₀. After incubation for 3 days at 22°C, the inoculated leaves were harvested. Same size leaf disc was taken from each leaf and was washed twice in sterile water and homogenized in 1 ml 0.9% NaCl. The samples were vortexed and serial dilutions prepared in 0.9% NaCl were plated on NYGA solid medium containing rifampicin (50 µg/ml) and kanamycin (25 µg/ml), and viable colonies were counted after 2 d of growth at 28°C. The study was conducted with four biological replications. Two factor ANOVA was used to compare different treatments. To determine which of the eight treatments differ from each other, Fisher’s least significant difference (LSD) comparisons were performed at P value of 0.05.

Bacterial inoculations and GUS assays of transgenic Arabidopsis plants

Pst DC3000 and Pst DC3000 carrying the AvrRpt2 gene were used for inoculation experiments. The pathogen was grown in NYGA liquid medium containing rifampicin (50 µg/ml) and kanamycin (25 µg/ml) as described above. The leaves of (i) the Arabidopsis npr1-1 mutant carrying the BGL2-GUS fusion gene or (ii) the npr1-1 mutant plants carrying the BGL2-GUS and transformed with either GmNPR1-1 or GmNPR1-2 were infiltrated with either Pst DC3000 or Pst DC3000 carrying the AvrRpt2 gene (10⁵ cfu/ml (OD₆₀₀ = 0.002). The inoculated leaves were harvested three days after infiltration and stained with X-gluc to localize the GUS activity [46].

Induction of the PR-1 gene transcription in Arabidopsis and soybean

Three-week old Arabidopsis npr1-1 (BGL2-GUS) mutant or npr1-1 (BGL2-GUS) transgenic plants containing either GmNPR1-1 or GmNPR1-2 were uprooted from the soil and washed in water. Roots were dipped in 20 ml ddH₂O in a 100 × 15 mm Petri dish for 24 h and then water was
replaced with 0.5 mM INA for 24 h [17]. Following INA feeding, seedlings were frozen in liquid nitrogen and stored at -80°C until preparation of RNAs. Total RNAs from individual samples were isolated using the Qiagen RNaseasy Plant Mini kit (Qiagen, Valencia, CA). RNA concentration was measured using a Unico spectrophotometer (Unico, Dayton, NJ). The same protocol was used for feeding Williams 82 seedlings with INA for various hours in Erlenmeyer flasks.

**Systemic induction of PR-1 in soybean**

Williams 82 seedlings were grown in trays containing soil. One-week old seedlings were stem-inoculated with the mycelia of *P. sojae* race 4 [47]. Uninoculated and trifoliate leaves, and *P. sojae*-infected tissues were harvested at 0, 1, 2, 3, 4, 5, 6, 9, and 14 days post inoculation, frozen in liquid nitrogen and stored at -80°C until their use for RNA isolation.

**RNA gel blot analysis**

Approximately 30 μg total RNAs per sample were fractionated by electrophoresis in 1% formaldehyde-agarose gels and blotted onto Zeta-Blot@ GT nylon membranes (Bio-Rad, Hercules, CA) as described earlier [48]. A soybean PR-1 gene, *GmPR1* (AI930866) [12] was used as a probe for the northern blot analyses of soybean RNA samples. The Arabidopsis *PR1* probe (NM_127025) was PCR amplified from Arabidopsis genomic DNA for northern analyses of Arabidopsis RNA samples. The probes were labeled with α-32P (dATP) [41]. Hybridization was carried out at 42°C for 16 to 18 h in buffer used for DNA gel blot hybridization. Membranes were washed twice for five min each in 2× SSC at room temperature followed by three times in washing buffer containing 2× SSC and 0.1% SDS at 65°C for 30 min each before exposure to the X-ray films.

**Authors’ contributions**

DS carried out SAR experiment in soybean and Arabidopsis, isolated cDNA clones, conducted GUS assays and sequence alignments, participated in designing experiments and drafting the manuscript. IMT carried out DNA sequence alignments, participated in designing experiments. MKB participated in designing and conducting research activities and drafting and finalizing the manuscript. All authors read and approved the final manuscript.

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**Additional material**

**Additional file 1**

Micro-colinearity between homoeologous regions containing *GmNPR1*-like sequences. mVISTA (http://genome.lbl.gov/cgi-bin/Vis
talInput?num_seqs=2; [49]) program was used to determine the micro-colinearity between Scaffold-159 and Scaffold-213 carrying *GmNPR1*-1 and *GmNPR1*-2, respectively. The location of the *GmNPR1* sequences is shown with a black box. The extent of identity between conserved sequences at the *GmNPR1* region is around 70%.

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**Additional file 2**

Micro-colinearity between homoeologous regions containing *GmNPR1*-1-like sequences. mVISTA (http://genome.lbl.gov/cgi-bin/Vis
talInput?num_seqs=2; [49]) program was used to determine the micro-colinearity between Scaffold-15 and Scaffold-90 carrying *GmNPR1*-1-like-1 and *GmNPR1*-1-like-2, respectively. The location of the *GmNPR1*-like sequences is shown with a black box between 32 and 34 kb sequence of Scaffold_15, which was the sequence 1 in the mVISTA analysis. The extent of identity between conserved genic sequences is around 70%.

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**Additional file 3**

Comparison of Soybean NPR1-like sequences with *Arabidopsis* NPR1. *GmNPR1*-1-like sequence (BE001977.1) was used to identify two *GmNPR1*-like peptides, *GmNPR1*-like-1 (Gm010500979.1:peptide; http://www.phytozome.net/cgi-bin/phytozome?name=scaffold_15:880000998802528) and *GmNPR1*-like-2 (Gm0090x00318:peptide; http://www.phytozome.net/cgi-bin/phytozome?name=scaffold_90:22819522760221) from Scaffold_15 and Scaffold_90 of the soybean genome sequence, respectively (http://www.phytozome.net/). ClustalW analysis ([http://www.ebi.ac.uk/clustalw/index.html](http://www.ebi.ac.uk/clustalw/index.html); [50]) revealed that these two peptides along with *GmNPR1*-1, *GmNPR1*-2, NPR3 (NP_199324.2), NPR4 (NP_193701.2) do contain the Cys216 residue (red font) essential for oligomerization of NPR1 (NP_176610.1).

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