Arabidopsis nonhost resistance gene *PSS1* confers immunity against an oomycete and a fungal pathogen but not a bacterial pathogen that cause diseases in soybean

*Sumit et al.*
Arabidopsis nonhost resistance gene *PSS1* confers immunity against an oomycete and a fungal pathogen but not a bacterial pathogen that cause diseases in soybean

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**Abstract**

**Background:** Nonhost resistance (NHR) provides immunity to all members of a plant species against all isolates of a microorganism that is pathogenic to other plant species. Three *Arabidopsis thaliana* PEN (penetration deficient) genes, PEN1, 2 and 3 have been shown to provide NHR against the barley pathogen *Blumeria graminis f. sp. hordei* at the prehaustorial level. Arabidopsis pen1-1 mutant lacking the PEN1 gene is penetrated by the hemibiotrophic oomycete pathogen *Phytophthora sojae*, the causal organism of the root and stem rot disease in soybean. We investigated if there is any novel nonhost resistance mechanism in Arabidopsis against the soybean pathogen, *P. sojae*.

**Results:** The *P. sojae* susceptible (*pss*) 1 mutant was identified by screening a mutant population created in the Arabidopsis pen1-1 mutant that lacks penetration resistance against the non adapted barley biotrophic fungal pathogen, *Blumeria graminis f. sp. hordei*. Segregation data suggested that PEN1 is not epistatic to PSS1. Responses of pss1 and pen1-1 to *P. sojae* invasion were distinct and suggest that PSS1 may act at both pre- and post-haustorial levels, while PEN1 acts at the pre-haustorial level against this soybean pathogen. Therefore, PSS1 encodes a new form of nonhost resistance. The pss1 mutant is also infected by the necrotrophic fungal pathogen, *Fusarium virguliforme*, which causes sudden death syndrome in soybean. Thus, a common NHR mechanism is operative in Arabidopsis against both hemibiotrophic oomycetes and necrotrophic fungal pathogens that are pathogenic to soybean. However, PSS1 does not play any role in immunity against the bacterial pathogen, *Pseudomonas syringae pv. glycinea*, that causes bacterial blight in soybean. We mapped PSS1 to a region very close to the southern telomere of chromosome 3 that carries no known disease resistance genes.

**Conclusions:** The study revealed that Arabidopsis PSS1 is a novel nonhost resistance gene that confers a new form of nonhost resistance against both a hemibiotrophic oomycete pathogen, *P. sojae* and a necrotrophic fungal pathogen, *F. virguliforme* that cause diseases in soybean. However, this gene does not play any role in the immunity of Arabidopsis to the bacterial pathogen, *P. syringae pv. glycinea*, which causes bacterial blight in soybean. Identification and further characterization of the PSS1 gene would provide further insights into a new form of nonhost resistance in Arabidopsis, which could be utilized in improving resistance of soybean to two serious pathogens.

**Keywords:** *Phytophthora sojae* susceptible (*pss1*), Sequence-based polymorphic (SBP) marker, *Fusarium virguliforme*, *Phytophthora sojae*, *Pseudomonas syringae pv. glycinea*
Background

Plants are exposed to an innumerable number of pathogenic organisms on a daily basis. However, because of immunity mechanisms only a few pathogens can infect and cause diseases in a particular crop species. One of the less understood immunity mechanisms is nonhost resistance (NHR), exhibited by all members of a plant species against non adapted pathogens [1,2]. The main NHR mechanisms were thought to be 1) incompatibility of non adapted pathogen with the physiology of nonhost plants and 2) inability of non adapted pathogens to overcome the plant defenses [3]. The first gene known to confer Arabidopsis NHR against a non adapted bacterial pathogen, Pseudomonas syringae pv. phaseolicola, is NONHOST1 (NHO1) which encodes a glycerol kinase [4,5]. NHO1 has also been shown to play an important role in the expression of gene-specific resistance against a bacterial pathogen [4].

NHR acts in two layers against the biotrophic fungal pathogens [6,7]. The first layer of NHR suppresses the invasion by non adapted pathogens at the pre-haustorial level. Three NHR genes, PEN1, PEN2 and PEN3, required for penetration resistance of Arabidopsis against the non adapted barley biotrophic fungal pathogen, Blumeria graminis f. sp. hordei have been isolated [6-8]. These genes act at the prehaustorial stage of the pathogen invasion [9]. PEN1 encodes a soluble N-ethylmaleimide sensitive attached receptor (SNARE) protein, which is involved in vesicle fusion and exocytosis of toxic compounds to the pathogen infection sites [8]. PEN2 encodes a glycosyl hydrolase, which has been localized to the peroxisomes [6]. PEN3 encodes an ATP-binding cassette (ABC) protein of the plasma membrane [7]. Cytological studies have demonstrated that PEN2 and PEN3 work together to generate and transport toxic chemicals into the infection sites [10]. The first layer of NHR prevents the biotrophic fungal pathogens from penetration and development of feeding structures, haustoria. Fungal pathogens that overcome the first layer of NHR encounter a post-haustorial defense mechanism. Some of the genes involved in the second layer of NHR in Arabidopsis are EDS1, PAD4 and SAG101 that are involved in plant defenses [6]. Downstream antagonistic defense pathways regulated by salicylic acid (SA) and the jasmonic acid (JA) are activated upon infection with biotrophic and necrotrophic pathogens, respectively [11]. SA and JA pathways are shown to be involved in the expression of nonhost resistance against the cowpea rust, Uromyces vignae, in Arabidopsis [12]. Similarly, studies of mutants lacking PEN1, 2, and 3 established that SA and JA pathways are also involved in the expression of nonhost resistance in Arabidopsis against the soybean pathogen Phytophthora sojae which causes the Asian soybean rust [13].

Recognition of pathogen associated molecular patterns (PAMPs) of non adapted pathogens by PAMP recognition receptors (PRRs) triggers the PAMP-triggered immunity (PTI) in nonhost species [14]. Recent studies have shown that PTI plays a major role in NHR [15]. Both chemical and physical barriers induced by PTI restrict non-adapted pathogens from invading nonhost species. Physical barriers include callose deposition at the infection sites and preformed barriers such as waxy coating on leaves. Chemical barriers include deposition of various reactive oxygen species (ROS) such as hydrogen peroxide and phenolic compounds at the infection site [16,17].

The plant responses to pathogenic invasions can be classified into two broad groups, PTI and the effector-triggered immunity (ETI) activated by strain-specific effectors. Both PTI and ETI play roles in providing nonhost resistance of plant species against non-adaptive or nonhost pathogens. It is speculated that PTI and ETI play an increasingly major and a minor role, respectively, in conferring nonhost resistance as the evolutionary distance between the nonhost and the nonhost pathogen species widens [18]. Conversely, ETI and PTI play an increasingly major and a minor role, respectively, in expression of nonhost resistance as the evolutionary distance between the nonhost and nonhost pathogens reduces.

Soybean (Glycine max L. Merr.) is one of the most important oil seed crops, a major source of livestock feed and an important biodiesel crop. Unfortunately, soybean is also a host of many pathogens that cause several serious diseases resulting in an estimated annual yield loss of $2.26 billion dollars [19]. In the United States, the estimated annual soybean yield losses just from the oomycete pathogen, P. sojae, have been valued to be over 300 million dollars [19]. Although various Rps (resistance to P. sojae) genes are utilized in generating Phytophthora resistant soybean cultivars [20,21], resistance conferred by these genes is effective only against a set of P. sojae races and is not durable. Partial resistance governed by quantitative trait loci (QTL) confers broad-spectrum resistance against P. sojae races in soybean. However, the level of partial resistance is not adequate enough to prevent significant crop losses [22]. Thus, it is essential to identify and use NHR mechanisms to provide soybean with broad-spectrum and durable resistance against this pathogen. As a first step towards achieving this goal, we have applied a forward genetic approach to identify and map the Arabidopsis thaliana NHR gene, PSS1, which provides resistance against the oomycete pathogen P. sojae. PSS1 is also required for immunity of Arabidopsis against the fungal pathogen, Fusarium virguliforme that causes the sudden death syndrome (SDS) in soybean.
Results

Arabidopsis pen1-1 mutant, but not nho1 mutant, is penetrated to single cells by the soybean pathogen P. sojae

Arabidopsis nho1 and pen1-1 mutants are defective in NHR mechanisms against the bacterial pathogen, Pseudomonas syringae pv. phaseolicola [5] and the powdery mildew fungus, Blumeria graminis f. sp. hordei [8], respectively. We investigated if the soybean pathogen P. sojae infects either of the two mutants. Ten-day-old seedlings grown in autoclaved double distilled water were inoculated with P. sojae zoospore suspensions and incubated for two days in the dark at 22°C. The inoculated seedlings were then stained with trypan blue dye and observed under a light microscope [23]. The pathogen did not penetrate either the wild-type ecotype Columbia-0 (Col-0) or the nho1 mutant (Figures 1A and B). P. sojae however penetrated single cells in pen1-1 (Figure 1C). These results indicated that in the pen1-1 mutant, the pre-haustorial NHR against P. sojae is compromised.

Identification of Phytophthora sojae susceptible (pss) putative mutants

We mutagenized pen1-1, compromised in pre-invasive immunity against P. sojae, with ethyl methane sulfonate (EMS) to identify mutants that are compromised in post-invasive immunity mechanisms. Over 3,500 M1 plants were planted and M2 seeds of these plants were harvested individually. Three hundred and seventy-nine randomly selected M2 families were grown to score for the chlorophyll mutants, a marker for determining the extent of EMS-induced mutation. About 5% of the families segregated for albino plants (Additional file 1), which suggested that the mutant population contained sufficient random point mutations and was suitable for screening. Approximately ≥70 seedlings of each M2 family were grown aseptically in 24-well microtiter plates in sterile water at 22°C for 10 days before inoculating with P. sojae zoospores. Following inoculation, seedlings were incubated for two days at 22°C in the dark, and then seedlings were stained with trypan blue for identifying putative mutants via staining of dead infected cells [23]. From screening 3,500 M2 families, we identified 30 putative mutants that were penetrated by P. sojae to multiple cells. The putative mutants were named as Phytophthora sojae susceptible 1 (pss1) through pss30. Subsequently, a detached leaf inoculation technique, previously reported for soybean, was applied in screening the putative mutants to identify the homozygous mutant plants [24]. We have applied a mapping approach in classifying these putative mutants. A homozygous mutant M4 family

![Figure 1](http://www.biomedcentral.com/1471-2229/12/87)
(0.2B17aLr-24) of the putative mutant pss1 showing complete loss of both pre- and post-haustorial NHR against P. sojae was selected. In successive generations, the selected pss1 mutant family was consistently infected by P. sojae. This mutant was phenotypically different from the pen1-1 because death in the mutant seedlings occurs in multiple cells as compared to in single cells in the pen1-1 mutant (Figure 1D, E, F, G, H). Although the P. sojae zoospores germinated and were able to form appressoria at the infection site, its growth was arrested immediately following germination on wild type Col-0 leaves. The pen1-1 mutant showed occasional death in single cells following P. sojae infection.

To determine the extent of P. sojae infection in inoculated tissues, detached pss1 leaves were collected 6 hours post inoculation (hpi) with P. sojae zoospore suspensions or treatments with water droplets. Leaves were then stained with aniline blue and the ultraviolet epifluorescence was visualized using a Zeiss Axioplan II compound microscope [25]. Extensive colonization by the pathogen was observed in the pss1 mutant (Figure 2A). Aniline blue stains the callose deposition and papillae formation and can be used to visualize fungal structures such as runner hyphae [26,27]. Callose deposition and papillae formation have previously been used as markers for attempted penetration by fungal pathogen [7]. Following inoculation with P. sojae zoospores, pss1 leaves showed extensive callose deposition and papillae formation across the infected leaf tissue as compared to pen1-1 and Col-0 (Figure 2A). Neither callose deposition nor papillae formation was detected in detached leaves that were treated with water droplets (Additional file 2A). At 6 hpi, extensive growth of the secondary hyphae was observed in P. sojae infected leaves of pss1 but not that of Col-0 and pen1-1 (Figure 2A).

To determine if P. sojae became adapted to the Arabidopsis pss1 mutant, we conducted microscopic study of the diseased lesions of the detached pss1 leaves 7 days post-inoculation (dpi) with the zoospore suspensions of the oomycete (Figure 2B). We observed enhanced hyphal growth and formation of reproductive structures, sporangia and oogonia on pss1 leaves (Figure 2B, Additional file 2B). Thus, we conclude that a gene mutated in pss1 is crucial for pre- and post-invasive nonhost immunity of Arabidopsis against the soybean pathogen, P. sojae. We named this gene PSS1.

Arabidopsis ecotypes showed leakiness in their NHR responses to P. sojae

Columbia-0 (Col-0) and Landsberg erecta (Ler) are the two most well-characterized ecotypes of Arabidopsis thaliana for mapping and gene cloning experiments [28,29]. We investigated if the ecotype Ler was completely immune to P. sojae so that it could be crossed to pss1 for generating mapping populations. However, Ler showed leakiness in its immune response against P. sojae and a significant proportion (12.5%) of the Ler seedlings were infected by P. sojae (Table 1). This result is not very surprising because the Arabidopsis ecotype L. erecta has recently been found to show susceptibility to another oomycete pathogen, Pythium irregulare [26]. We therefore inoculated 22 A. thaliana ecotypes with P. sojae zoospores and discovered that ecotypes, Bensheim, Nossen-0 (No-0) and Niederzenz-0 (Nd-0) were completely immune to the pathogen (Table 1). We selected Nd-0 for mapping experiments because it is morphologically similar to Col-0. Furthermore, a few molecular markers polymorphic between Nd-0 and Col-0 were already available [30].

PSS1 is required for nonhost resistance of Arabidopsis against P. sojae

Forty-two F2:3 families developed from the cross between pss1 and Nd-0 were evaluated for segregation of host responses to the pathogen infection. At least 24 progenies of each F2 plants were scored for disease phenotypes. The segregation of alleles at the PSS1 locus among the F2,3 families fit to the 1:2:1 genotypic ratio for a single gene model (p = 0.81; Table 2). This observation suggested that PSS1 is a single gene with no apparent epistatic effect from PEN1.

In addition to these 42 F2,3 families, we determined the phenotypes of additional families. In this experiment, only eight progenies per family were screened to identify the F2,3 families that carry pss1 in homozygous condition. To further confirm that PSS1 is a single gene with no epistatic effect from PEN1, we evaluated the segregation of the PEN1 alleles among 20 F2,3 families, homozygous for the pss1 allele, using the dCAPS marker for PEN1 alleles [7]. PEN1 alleles segregated in a 1:2:1 ratio (p = 0.67) among the 20 families, homozygous for the pss1 allele (Figure 3). This result suggested an independent segregation for the two genes. Among the 20 homozygous families for the pss1 allele, four were shown to carry the PEN1 allele in homozygous condition. If the PEN1 allele was epistatic to PSS1 and PSS1 were to encode only a post-invasive resistance mechanism, then the pen1-1 allele should have been in recessive homozygous condition among the pss1 homozygous families. Thus, PSS1 encodes a new form of penetration resistance.

Expression of P. sojae effector genes in pss1 during infection

To determine the extent of P. sojae-gene expression, we selected two effector genes to conduct RT-PCR. It has been shown that P. sojae carries over 370
candidate effector proteins containing N-terminal RXLR-DEER motifs [31]. We studied the expression of *PsAvh223* and *PsAvh224* [32] in *pss1*, *pen1-1* and *Col-0* following inoculation with *P. sojae*. Both effector *P. sojae* genes were highly expressed in the *pss1* mutant as compared to *pen1-1* and *Col-0* (Figure 4). This result indicates that the *P. sojae* colonized to a greater extent in *pss1* as compared that in *pen1-1* or *Col-0*.

**Figure 2** Responses of the *pss1* mutant following *P. sojae* infection. **A**, Leaves of 21 day old Col-0, *pen1-1* and *pss1* seedlings were inoculated with *P. sojae* zoospores and stained with aniline blue and visualized under a Zeiss Axioplan II compound microscope with ultraviolet epifluorescence [25]. (i) and (iv), Col-0; (ii) and (v), *pen1-1*; and (iii) and (vi), *pss1* leaves that were stained with aniline blue to detect callose deposition 6 hours post inoculation (hpi) with *P. sojae* by epifluorescence of the aniline blue. (i-iii), 50X magnification; and (iv-vi), 200X magnification. Arrows indicate sites of callose deposition (ca) and secondary hyphae (sh). The experiment was repeated twice with similar results. **B**, Leaves of 21 day old Col-0, *pen1-1* and *pss1* seedlings were inoculated with *P. sojae* zoospores and stained with trypan blue and visualized under a Zeiss Axioplan II compound microscope under bright field illumination [23]. (i) and (iv), Col-0; (ii) and (v), *pen1-1*; and (iii) and (vi), *pss1* leaves that were stained with trypan blue to detect cell death and fungal structures 7 days following inoculation with *P. sojae* zoospores. Arrows indicate reproductive structures, oogonia (oo), sporangia (sp) and secondary hyphae (sh), which were visible in infected *pss1* leaves. (i-iii), 100X magnification; and (iv-vi), 200X magnification. The experiment was repeated twice with similar results.
In order to map the PSS1 gene, we applied bulked segregant analysis (BSA) [33]. Four bulks of P. sojae susceptible plants each carrying 7–8 F_{2:3} susceptible families and one bulk of P. sojae resistant plants containing two homozygous (PSS1PSS1) and six heterozygous (PSS1pss1) F_{2:3} families were generated. These five bulks and Col-0 and Nd-0 were included in BSA. We used sequence-based polymorphic (SBP) [34], SSLP and CAPS markers in conducting BSA.

The PSS1 region was putatively mapped to the south arm of chromosome 3 (Figure 5A). To develop a high-density map of the PSS1 region, five SBP markers from this region were generated. SBP_20.71 marker showed a recombination event with the PSS1 locus in the F_{2:3} family 93 suggesting that PSS1 is located south of this marker (Figure 5B). No recombination was observed between PSS1 and SBP_23.46 marker, located at the telomeric end of chromosome 3 (Figure 5C). The physical distance between SBP_20.71 and SBP_23.46 is ~2.75 Mb.

The Arabidopsis pss1 mutant is infected by the fungal pathogen, Fusarium virguliforme, which causes sudden death syndrome in soybean

We investigated if PSS1 controls Arabidopsis NHR against the fungal pathogen, F. virguliforme that causes sudden death syndrome (SDS) in soybean. From the segregating materials used for mapping the PSS1 gene, we selected six F_{2:3} families that were homozygous for either PSS1 or pss1 alleles (Additional file 3) and used these families in determining the role of PSS1 in NHR of Arabidopsis against F. virguliforme. Seedlings of the selected

### Table 1 Responses of Arabidopsis ecotypes to P. sojae

| Seedling Inoculation | Leaf Inoculation |
|----------------------|------------------|
| Ecotypes            | ¹Immune | ²Infected | % Infection | ¹Immune | ²Infected | % Infection |
| AUA/Rhon            | 42      | 0        | 0.00        | -       | -        | -          |
| Bensheim            | 45      | 0        | 0.00        | -       | -        | -          |
| Cape Verde-0        | 24      | 1        | 4.00        | 19      | 5        | 20.83      |
| Catania             | -       | -        | -           | 21      | 3        | 12.50      |
| Columbia-0          | 250     | 5        | 1.96        | 20      | 1        | 4.76       |
| Dal(1)              | -       | -        | -           | 17      | 7        | 29.17      |
| Ellershausen-0      | -       | -        | -           | 19      | 5        | 20.83      |
| Estland             | 19      | 2        | 9.52        | 14      | 4        | 22.22      |
| Greenville-0        | 11      | 1        | 8.33        | -       | -        | -          |
| Isenberg            | -       | -        | -           | 14      | 7        | 33.33      |
| Kaunas-0            | -       | -        | -           | 20      | 4        | 16.67      |
| Kendalville         | 53      | 1        | 1.85        | -       | -        | -          |
| Koln-59             | -       | -        | -           | 24      | 0        | 0.00       |
| Lanark-0            | -       | -        | -           | 10      | 8        | 44.44      |
| Landsberg erecta    | 348     | 15       | 4.13        | 28      | 4        | 12.50      |
| Le Mans-2           | -       | -        | -           | 19      | 5        | 20.83      |
| Limeport            | -       | -        | -           | 20      | 4        | 16.67      |
| Muhlen-0            | 29      | 0        | 0.00        | 20      | 4        | 16.67      |
| Niederenz-0         | 36      | 0        | 0.00        | 21      | 0        | 0.00       |
| Nossen-0            | 38      | 0        | 0.00        | -       | -        | -          |
| Oystese-0           | -       | -        | -           | 19      | 5        | 20.83      |
| Poppelsdorf-0       | -       | -        | -           | 20      | 4        | 16.67      |
| RLD1                | 30      | 1        | 3.23        | -       | -        | -          |
| S96                 | 37      | 1        | 2.63        | -       | -        | -          |

¹ No detectable host response after inoculation with P. sojae spores. ² Visible necrosis at the inoculation site was observed.

### Table 2 Segregation of Pss1 alleles among the F_{2:3} families derived from a cross between the pss1 mutant and the ecotype Nd-0

| Genotype                  | Observed | Expected |
|---------------------------|----------|----------|
| Homozygous resistant (Pss1/Pss1) | 12       | 10.5     |
| Heterozygous (Pss1/pss1)   | 21       | 21       |
| Homozygous susceptible (pss1/pss1) | 9        | 10.5     |
| Total                      | 42       | 42       |

χ² value: 0.43

P-value: 0.81
families were grown in 24-well microtiter plates for 10 days and then inoculated with *F. virguliforme* conidial spores. Infected seedlings were stained with trypan blue and observed under a light microscope (Figure 6A). Significant proportions of seedlings in six families carrying the *pss1* allele were infected by the fungal pathogen (Figure 6B). This result suggests that *PSS1* is also essential for NHR against the soybean pathogen, *F. virguliforme*.

**PSS1** is not required for NHR of Arabidopsis against the non-adaptive pathogen *Pseudomonas syringae pv. glycinea* that causes bacterial blight in soybean  
We investigated if *PSS1* is required for NHR of Arabidopsis against the bacterial pathogen, *Pseudomonas syringae pv. glycinea* (*Psg*) that causes bacterial blight in soybean [35]. We inoculated the six *F₂₃* families homozygous for *pss1* and five *F₂₃* families homozygous for the *PSS1* allele with *Psg* (Figure 6C). We observed no association of *PSS1* and *pss1* alleles with the colony forming units (cfu) of the bacterial pathogen. We classified the responses of the selected families into two broad groups, one with cfu comparable to those observed for Col-0 and Nd-0; and the other one with five- or more-fold lesser cfu as compared to those observed in Col-0 and Nd-0. Surprisingly, *pen1-1* consistently showed about 4-5-fold less bacterial growth as compared to that in Col-0 (Figure 6C). To determine if *PEN1* is required for growth of *Psg*, we genotyped the selected susceptible and resistant *F₂₃* families for the *PEN1* locus (Additional file 4). No association was observed between alleles at the *PEN1* locus and the levels of *Psg* cfu. These results suggested that an unknown mutation in the *pen1-1* genotype is most likely involved in enhancing resistance of Arabidopsis against *Psg* (Figure 6C) and the unknown gene could be a negative regulator of disease resistance.

**Discussion**
Transfer of NHR mechanisms across species may lead to development of broad-spectrum and durable resistance in economically important crop species. Identification of *NHO1* and *PEN* genes established the molecular basis of NHR. It also suggested the feasibility of transferring single gene-encoded NHR across plant species for creating durable and broad-spectrum resistance [4,6-8].

Here we have described the Arabidopsis *PSS1* locus that carries one of the nonhost resistance genes conferring immunity of Arabidopsis against two important soybean pathogens, *P. sojae* and *F. virguliforme*. Considering the disease phenotypes observed in detached leaves of *pss1* as opposed to that in detached leaves of the *pen1-1* mutant following *P. sojae* inoculation (Figures 1 and 2), the NHR mechanism governed by *PSS1* is most likely important not only to provide penetration resistance, but also to confer necessary protection against further spread of the pathogen. *pss1* supports secondary hyphal growth and sporulation of *P. sojae* (Figure 2). These observations suggest that *PSS1* encodes a NHR defense mechanism that regulates...
both penetration and post-penetration resistance. It has been shown that the NHR mechanism at the post-haustorial stage is most important in sow thistle for providing resistance against a poorly adapted powdery mildew fungus, *Golovinomyces cichoracearum* UMSG1 [36]. Similar mechanism could also be important for NHR of *Arabidopsis* against the non-adapted oomycete pathogen, *P. sojae*.

Segregation data from a cross between *pss1* and Nd-0 revealed 1:2:1 genotypic segregation ratio for the alleles at the *PSS1* locus (Table 2); and therefore, it is a single gene. Alleles at the *PEN1* locus segregated independently of the alleles at the *PSS1* locus (Figure 3). The *P. sojae* susceptible phenotype of the *pss1* allele is manifested even in the presence of *PEN1*. Thus, *PSS1* controls a novel defense mechanism for penetration resistance against the oomycete pathogen, *P. sojae* and the fungal pathogen, *F. viruliforme*. *PEN* genes have been shown to regulate two distinct NHR mechanisms that are involved in penetration resistance. Monogenic inheritance of *PSS1* with no epistatic effect from *PEN1* suggests that an additional *Arabidopsis* NHR mechanism is operative against penetration by oomycete and *Fusarium* pathogens. *PSS1* is located in an approximately 2.75 Mb region flanked by two sequence-based polymorphic markers, SBP_20.71 and the telomere-specific SBP_23.46 (Figure 5C). This region does not contain any characterized plant defense or disease resistance genes. Thus, most likely we have identified a novel nonhost resistance mechanism in *Arabidopsis*.

The important hallmarks of a successful adapted pathogen are its ability to establish feeding structures, derive nutrition from the host and finally to complete its lifecycle in the host plant [3]. Aniline blue staining has previously been used to show oomycete feeding structures such as runner hyphae [26]. We observed secondary hyphae even after 6 hpi suggesting that *P. sojae* is able to form feeding structures in *pss1* leaves at a very early stage following inoculation (Figure 2A). Sporangia are specialized asexual reproductive structures of oomycetes which can either germinate into hyphae or release about 10–30 zoospores.

![Figure 5 Molecular mapping of the PSS1 locus. A, Identification of SSLP markers linked to PSS1. Similar amplification patterns of SSLP markers CIW20 and CIW22 in susceptible bulks (S1, S2, S3 and S4) and Col-0 suggested that PSS1 is putatively linked to the two markers. Amplification patterns of a distantly mapped SSLP marker, LUGSSLP08 in the bulked DNA samples are shown as the control. B, Co-segregation of PSS1 with six molecular markers of the south arm of chromosome 3. Twenty-two susceptible F2:3 families except one, F2:3 family 93, showed same amplification patterns as in Col-0 for these markers. F2:3 family 93 showed recombination between PSS1 and SBP_20.71. C, Molecular map of the PSS1 region. Five SBP markers were developed for the PSS1 region that was mapped to the southern arm of chromosome 3.](http://www.biomedcentral.com/1471-2229/12/87)
to complete the asexual life-cycle. The male and female reproductive structures, antheridia and oogonia, are fused to develop oospores and complete the sexual life [37]. 

*P. sojae* developed both sporangia and oogonia in infected *pss1* leaves; and thus, completed its life cycle in this mutant (Figure 2B). In contrast, in *pen1-1* leaves the pathogen
was able to penetrate single cells, which die following penetration; while in the wild type Col-0 leaves, germinated P. sojae zoospores failed to penetrate host cells (Figure 2B).

Lack of epistasis of PEN1 on PSS1 (Figure 3), growth of secondary hyphae and rapid induction of effector genes in the pss1 mutant, and most importantly completion of the P. sojae life cycle in infected pss1 mutant leaves suggest that PSS1 encodes a novel NHR mechanism that regulates both pre- and post-invasive resistance of Arabidopsis against the nonhost pathogen. Transfer of this to soybean could play an important role in creating broad-spectrum resistance against the nonhost pathogen. PEN1 acts at the pre-haustorial level. Identification and further characterization of the gene would provide us further insights about this new form of nonhost resistance against two non-adaptive soybean pathogens. This study thus laid the foundation for possible development of soybean germplasm with durable resistance against two serious pathogens.

Methods

Mutagenesis of pen1-1

About 15,000 pen1-1 seeds were divided into three lots of ~5,000 seeds each. The three seed lots were then treated with 0.2%, 0.25%, and 0.3% EMS solution, respectively, for 15 h. The mutants were classified into three groups based on the concentration of EMS used for mutagenesis. Seeds were thoroughly washed 8 times in tap water and left in water on shaker for an additional hour. On an average, 1,000 seeds were sown on each flat (10-1/2” x 20-7/8”). Two weeks later plants were transplanted to trays containing 32 pots. The M1 plants were selfed and seeds of 3,556 M2 families were individually harvested.

Inoculation methods and disease scoring

Two methods of inoculation were applied: i) seedling inoculation and ii) detached leaf inoculation. For the seedling inoculation, more than 70 A. thaliana seeds of individual M2 families were sterilized in the wells of 24-well microtiter plates (Costar® Corning Inc., Corning, NY) by first soaking in 70% ethanol for about 5 minutes and then washing with 50% Clorox bleach and 0.05% Triton X-100 for 10–15 minutes. The seeds were later rinsed four times with autoclaved water to remove any traces of bleach and/or ethanol. The seeds were then soaked aseptically in 300 μl autoclaved, double distilled water and incubated at 4°C for 48 h followed by incubation at 22°C for 10 days under constant light (100 μE/m²/s). Seedlings were then inoculated with 300 μl P. sojae zoospores race 25 (10⁵ zoospores/ml). After two days of incubation at 22°C in the dark, the inoculated seedlings were stained with trypan blue and then destained with saturated chloral hydrate for 48 h [23]. Destained seedlings were mounted on a glass slide in 50% glycerol and observed under a Zeiss microscope (Zeiss Incorporated, Thornwood, NY) and seedlings showing enhanced cell death in multiple cells were scored as susceptible.

For the leaf inoculation, the seeds were sown on LC1 soil-less mixture (Sun Gro Horticulture, Bellevue, WA) under a 16 h light/8 h dark regime at 21°C with approximately 60% relative humidity. The light intensity was maintained at 120–150 μE/m²/s [41]. Ten days after sowing, the seedlings were transplanted into a
new LC1 mixture. The newly transplanted seedlings were covered with humidity domes for two days and thereafter watered every fourth day. A fertilizer mixture of 15:15:15::N:P:K (1% concentration v/v) was applied to the seedlings seven days after transplantation.

Three leaves (leaf # 4, 5 and 6 from the apex) were detached from 21-day old plants and placed on moist Whatman filter papers, in Petri dishes. Each leaf was then inoculated with 10 μl of \( P. \text{sojae} \) zoospore suspensions (10^5/ml). The Petri dishes, following closing the lids, were incubated under constant light (50 μE/m^2/s) at 22°C. The inoculated plants were scored 48 and 72 h post inoculation (hpi) for resistant and susceptible host responses. In some experiments, 10-μl droplets of autoclaved double distilled water were placed on the surface of detached leaves as a negative control.

**Microscopic evaluations**

Leaves of 21-day old Arabidopsis wild type Col-0, pen1-1 and pss1 mutant plants were inoculated with \( P. \text{sojae} \) spores (1.0 x 10^5 spores/ml) and stained with trypan blue 7 days post inoculation (dpi) [23] and with aniline blue dye at 6 hours post inoculation (hpi) [25]. The stained leaves were mounted in saturated chloral hydrate for trypan blue dye [23] or in 70% glycerol and 30% aniline blue solution (0.01%) for aniline blue dye [25]. Stained images were examined using a Zeiss Axioplan II compound microscope equipped with AxioCam color digital camera.

**DNA preparation, PCR and BSA**

Arabidopsis genomic DNA was extracted by CTAB method [42]. Young inflorescence or a rosette leaf was selected for DNA extraction. Equal amount (10 μg) of DNA from individual F2:3 families were mixed to obtain bulk DNA samples. The final DNA concentration of these bulk DNA samples for PCR was 20 ng/μl. The PCR reaction mixtures contained 2 mM MgCl2 (Bioline, Taunton, MA), 0.25 μM each of forward and reverse primer (Integrated DNA Technologies, Inc., Coralville, Iowa), 2 μM dNTPs and 0.5 U Choice Taq polymerase (Denville Scientific, Inc., Metuchen, NJ). For SSLP markers, PCR was conducted at 94°C for 2 min, and then 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. Finally, the mixture was incubated at 72°C for 10 min. For the CAPS markers, PCR was conducted at 94°C for 2 min, and then five cycles of 94°C for 30 s followed by decreasing annealing temperatures from 55°C to 50°C.

**Table 4 List of CAPS markers polymorphic between Arabidopsis ecotypes Col-0 and Nd-0**

| CAPS marker | 1Restriction enzyme | 2Primers |
|-------------|---------------------|---------|
| 1H1L-1.6    | RsaI, Tsp509I       | F:CTAGAGCTTGAAGTTGATG  
R:TGTGGTCTTCTGCTTCTG |
| 20B4L-1.6   | DdeI                | F:CTAAGATGGGAATGTTGGG  
R:GAACCTATGTTAGTACC |
| 40E1T7      | Accl                | F:GGTCCACTTGTTCAAGATG  
R:RGCAAGCCATGAAACAG |
| AF2         | DdeI                | F:TCGGCTTTTGGTCTTTTTCTA  
R:CCATCATTAGGCGGATTTT |
| B9-1.8      | Tagl                | F:CATCTGCAAGATGCTCTCCAG  
R:RGTATCGCCATATTCTCAGTC |
| CAT         | Tsp509I             | F:GACCGATGAAAGAGATCCATACTGCG  
R:RCACAGTCACTGGCAGTAAGCT |
| ER          | Ddel                | F:GAATTCTATGCTGTTTCAAGTCG  
R:RCAATACTGAGTCGAGTAATC |
| G4026       | Rsol                | F:GTACGGGTCTCTGCTCTCTTA  
R:GGGGGTAGCAGTTCAGAGCAG |
| G4711       | Ddel                | F:CCTGTGAAAAACCGCGTCGTCATT  
R:RCAACAACTTGGGCTGCGACTTC |
| GPA1.1      | Tsp509I             | F:ATTCTGTCGTCTCATTACTC  
R:GGGGGATTGAGTAAAGGGAGA |
| JM411       | Ddel                | F:GCAAAACACTAAGAAGTT  
R:RGTCTGACCTGCAAAGAT |
| LFY3        | Rsol                | F:GACGGGCTGCTGAAAGTT  
R:RGAATTTGATTTTCAAGTC |
| Mi342       | Tsp509I             | F:GAAGTACAGCGGGTCAAAAAAGAG  
R:RTGCTGCGCTGTTATACCACTATG |
| M555        | Accl                | F:CCCCAAATTTGATATACTCAATTAC  
R:RGTCTGCAATTTAAATGTCAGT |
| M59         | Rsol, Tsp509I       | F:GTGCATGATATGATCGAC  
R:RGAATTTGATTTTCAAGTC |
| MBK23A      | Tagl                | F:GATACATAGCGGCTGCTGCTG  
R:RATTACAGCGGCTGCTGCTG |
| PA1.1       | Rsol, Tsp509I       | F:GATCCAAATTTGATATACTGAT  
R:RGCAGAATTGATTTTCAAGTC |
| T20D161     | Tsp509I             | F:CGATTGCTGCTGTTTCAAGTC  
R:RATGCTGCAATTTAAATGTCAGT |
| T6PS-4.8    | Rsol                | F:TGAAGACACCGCTGCTGCTGCTG  
R:RCAACAACTTGGGCTGCGACTTC |

1Restriction endonucleases used for individual CAPS markers are shown.

2Primers: F, forward primer; R, reverse primer
(−1°C/cycle) and 72°C for 1 min. Then 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min were conducted. Finally, the reaction mixture was incubated at 72°C for 10 minutes. PCR was carried out in PTC-100 Programmable Thermal Controllers (MJ Research Inc.). The amplified products were resolved on a 4% agarose gel by running at 8 V/cm. The ethidium bromide stained PCR products were visualized following illumination with UV light.

RNA isolation and RT-PCR experiments
Total RNA was isolated from leaf tissues using the TRIzol® reagent according to the manufacturer’s instructions (Invitrogen, Inc., Carlsbad, CA). RNA samples were treated with DNase I (Invitrogen, Inc., Carlsbad, CA) to eliminate any DNA contamination [43]. cDNAs were prepared according to manufacturer’s recommendations (Invitrogen, Inc., Carlsbad, CA). cDNAs were amplified using specific primers (Table 3) were used to PCR amplify cDNA fragments from these samples. RT-PCR was conducted for the above genes using the cDNAs prepared from infected leaves at 1 d and 3 d post inoculation or treatment with UV light. The ethidium bromide stained PCR products were visualized following illumination with UV light.

### Table 5 Sequence Based Polymorphic (SBP) markers generated for the PSS1 region

| Name    | Primer             | Enzyme | Amplicon Size (bp) |
|---------|--------------------|--------|--------------------|
| SBP_22.95 | GAGGCTCCGTTACTC   | RsaI   | 309                |
|          | TTACTG R: CGACGAGCTTC |        |                    |
| SBP_22.98 | CGACGTCAACTCTCC   | TaqI   | 230                |
|          | GTTA R: CCGATGATGGA GAAGAAAAA |      |                    |
| SBP_23.06 | AAATGGGGAACACCA  | Tsp509I| 180                |
|          | ACAAA R: GCTTCTCCGT GGAAGAAGAT |       |                    |
| SBP_23.09 | TCGGATGATCCTTCC   | TaqI   | 235                |
|          | TTCA R: GCTITTCCCGA AAATGGGATA |     |                    |
| SBP_23.46 | GACCAAAATGTCTCTG A | TaqI   | 520                |
|          | GATGTC R: ACCCAAGG CGGTGTGGGAAAG |   |                    |

*Primers: F, forward primer; R, reverse primer. *Restriction endonucleases used for individual CAPS markers are shown.

Molecular markers

### Table 5 Sequence Based Polymorphic (SBP) markers generated for the PSS1 region

Sequencing of primers for SSLP markers were obtained from The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org). SSLP markers, polymorphic between Col-0 and Nd-0, were selected to cover the entire genome with a density of one SSLP marker/2 Mb DNA. For the SSLP-thin regions, CAPS and SSLP markers were designed [34]. The primers for the CAPS are presented in Table 4 and that for the SBP markers are presented in Table 5.

### For inoculation of F2:3 with F. virguliforme

For inoculation of F2:3 families with F. virguliforme, more than 70 seedlings of each family were grown in 24-well microtiter plates (Costar® Corning Inc., Corning, NY) as described earlier. The seedlings of individual family were then inoculated with about 300 μF. virguliforme spores (106 spores/ml) and incubated in the dark for 48 h. The inoculated seedlings were then stained with trypan blue dye as previously described and observed under a microscope (Zeiss Inc., Thornwood, NY). Seedlings showing enhanced cell death in multiple cells were scored as susceptible.

### For leaf inoculation of F2:3 with the bacterial pathogen, P. syringae pv. glycinea

For leaf inoculation of F2:3 with P. syringae pv. glycinea, Arabidopsis plants were grown in a 10 h light/14 h dark period at 21°C under light intensity of 100–120 μmol/ cm²/sec. P. syringae pv. glycinea was grown on King’s B medium containing rifampicin (100 μg/ml) at 28°C. For liquid culture, bacteria were grown in liquid King’s B medium without rifampicin at 25°C for 24 h. Four-week old plants were leaf inoculated with bacterial suspensions with 0.10 OD600nm (~2 x 106 cfu/ml) diluted in 10 mM MgCl2 solution [44]. Four leaves of each plant were inoculated on the abaxial side with 50 μl bacterial suspensions using the blunt end of a 1 ml syringe (BD, Franklin Lakes, NJ). Plants were then covered with a humidity dome until samples were harvested for plating. 1 cm diameter leaf discs from each inoculated leaf samples were harvested at 0 and 3 days post-inoculation. Leaf discs of eight leaves from two plants were pooled to make one replication and three biological replications were performed. Serial dilutions of the extracts from leaf disc samples were plated on King’s B medium containing rifampicin. Colony forming units (cfu) were counted 2 days following plating.

### Additional files

Additional file 1: EMS mutants created in Arabidopsis thaliana pen1-1 mutants showed chlorophyll-lacking mutants among 5% of the M2 families. The albino seedlings are shown with arrows.

Additional file 2: A: Autofluorescence of pss1 mutant leaf. Detached leaves of 21-day old seedlings of the pss1 mutant were mock inoculated with sterile water and stained with aniline blue and observed under ultraviolet epifluorescence 6 hours post inoculation. The image was taken at 50X magnification. The experiment was repeated three times with
similar results. B: The pss1 mutant is a host for soybean oomycete pathogen, P. sojae. Detached leaves of pss1 mutant were inoculated with P. sojae zoospores (10^3 spores/ml) and stained with trypan blue dye 7 days post inoculation (dpi). Formation of female reproductive structures, oogonia (oo) and asexual reproductive structures, sporangia (sp) indicate that the pathogen is able to complete its life cycle on the host pss1 mutant leaves, thus signifying a complete breakdown of Arabidopsis nonhost resistance in this mutant. Numbers indicate the approximate size of the reproductive structures, which is in close agreement with the average size of the reproductive structures of the Phytophthora genus [45].

**Additional file 3: Identification of F_{2,3} families homozygous for alleles at the PSS1 locus.** A. Inoculation of a 10 day old pss1 seedling with P. sojae spores following by staining with trypan blue dye showed extensive hyphal growth and subsequent cell death. Image (100X magnification) was taken at 2 dpi. B. The indicated section of A at a higher magnification. C. Reproses of 10-day old seedlings of six F_{2,3} families, homozygous for the pss1 allele (5-4 through S-434), and six F_{2,3} families, homozygous for the PSS1 allele (R194 through R332), were inoculated with P. sojae zoospores. Data are mean of percent seedlings infected from three independent experiments. Error bars indicate standard error (S.E.) among experiments.

**Additional file 4: Genotype of six P. sojae susceptible (pss1)pss1 (5-4 through S-434) and five resistant (PSS1PSS1) (R194 through R332) F_{2,3} families and the pss1 mutant for the PEN1 alleles.** A. homozygous for pen1-1, B, homozygous for PEN1; H, heterozygous.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RS, BB, MX and DS conducted the experimental work. RS and BBS wrote the first draft and contributed to writing the subsequent drafts of the manuscript. MNB conceived the research, designed the experiments and wrote the final draft of the manuscript. All authors read and approved the manuscript.

Acknowledgements

We thank Drs. Paul Schulze-Lefert and Jammin Zhou for providing the seeds of pen1-1and nzho mutants, respectively. We thank Dr. Coralee Lashbrook for providing seeds of Arabidopsis thaliana ecotypes. We thank Drs. Steve Rödermel and Yanhai Yin for providing primers for some of the SSLP and CAPS markers, respectively. We thank Dr. Jack Horner, Randall Den Adel and Ms. Tracey Pepper of the microscopy and nanoinaging facility at Iowa State University for assisting us with microscopic studies. This work was supported by a grant from the Consortium for Plant Biotechnology Research (CPBR) and Iowa Soybean Association.

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Received: 18 December 2011 Accepted: 23 May 2012

Published: 13 June 2012

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doi:10.1186/1471-2229-12-87

Cite this article as: Sumit et al: Arabidopsis nonhost resistance gene PSS1 confers immunity against an oomycete and a fungal pathogen but not a bacterial pathogen that cause diseases in soybean. BMC Plant Biology 2012, 12:87.