Short Title: Arabidopsis PSSI enhances soybean SDS resistance

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Arabidopsis novel glycine-rich plasma membrane PSS1 protein enhances disease resistance in transgenic soybean plants

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Author contributions:
M.K.B conceived and supervised the project; S.K.S. conducted the SHORE analysis, R.S. and B.S. cloned the PSS1 gene; R.S., B.B.S. and M.N.N. performed the construction of binary vectors for soybean transformation and harvested R1 seeds; B.W. investigated the gene function; B.W., B.B.S., R.S., M.N.N., S.S. and Y.Y. evaluated the transgenic soybean plants; B.W., M.N.N. and R.S. analyzed the data; B.W. and R.S. wrote the first draft and M.K.B completed writing the manuscript.

One-sentence summary: Arabidopsis nonhost resistance gene PSS1 encoding an unknown glycine-rich plasma membrane protein has shown to enhance sudden death syndrome resistance in transgenic soybean plants.

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Nonhost resistance is defined as the immunity of a plant species to all nonadapted pathogen species. Arabidopsis ecotype Columbia-0 (Col-0) is nonhost to oomycete plant pathogen *Phytophthora sojae* and fungal plant pathogen *Fusarium virguliforme* that are pathogenic to soybean. Previously, we reported generating *pss1* mutation in the *pen1-1* genetic background, genetic mapping and characterization of the Arabidopsis nonhost resistance *Phytophthora sojae* susceptible gene locus, *PSS1*. In this study, we identified six candidate *PSSI* genes by comparing single nucleotide polymorphisms (SNPs) of (i) the bulked DNA sample of seven F\textsubscript{2:3} families homozygous for the *pss1* allele and (ii) the *pen1-1* mutant with Col-0. Analyses of T-DNA insertion mutants for each of these candidate *PSSI* genes identified the *At3g59640* gene encoding a glycine rich protein as the putative *PSS1* gene. Later, complementation analysis confirmed the identity of *At3g59640* as the *PSS1* gene. *PSS1* is induced following *P. sojae* infection as well as expressed in an organ-specific manner. Co-expression analysis of the available transcriptomic data followed by reverse transcriptase PCR suggested that *PSS1* is co-regulated with *ATG8a* (*At4g21980*), a core gene in autophagy. *PSS1* contains a predicted single membrane spanning domain. Subcellular localization study indicated that it is an integral plasma membrane protein. Sequence analysis suggested that soybean unlikely contains a *PSS1*-like defense function. Following introduction of *PSS1* into the soybean cultivar ‘Williams 82’, the transgenic plants exhibited enhanced resistance to *F. virguliforme*, the pathogen that causes sudden death syndrome.
Nonhost resistance (NHR) is defined as immunity of an entire plant species against all races or isolates of a non-adapted pathogen species. Examples include fungi and oomycete pathogens that fail to penetrate and propagate in the nonhost plants (Heath, 2000; Mysore & Ryu, 2004; Lipka et al., 2005; Senthil-Kumar & Mysore, 2013; Hadwiger, 2015; Lee, Whitakeer & Hutton, 2016). It is widely considered that non-host resistance mechanisms are multilayered and are often elicited by pathogen-associated molecular patterns (PAMPs) (Jones & Dangl, 2006). Upon failure of the pathogens to invade a non-host due to the activation of basal host resistance triggered by PAMPs (PAMP triggered immunity; PTI), effectors are secreted by the phytopathogens for deriving nutrition and interfering the host defense physiology leading to development of susceptibility known as effector triggered susceptibility (ETS). Host plants then express cognate R genes encoding receptors that recognize one or more of these effectors and trigger immunity (effector triggered immunity; ETI), which is manifested commonly as hypersensitive reaction or programmed cell death (Jones & Dangl, 2006).

A mutant study identified *PENETRATION DEFICIENT 1* (*PEN1*), *PEN2*, *PEN3* genes that confer nonhost immunity of *Arabidopsis thaliana* ecotype Col-0 against the barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). Study of the three *PEN* genes revealed two parallel nonhost resistance mechanisms that suppress penetration of *B. graminis* f. sp. *hordei*. One mechanism, regulated by *PEN1*, entails vesicle-mediated secretion of free radicals such as hydrogen peroxide to invasion sites. In the other mechanism, *PEN2* and *PEN3* regulate transport of anti-microbial glucosinolates and tryptophan derived secondary metabolites to infection sites (Clay et al., 2009; Schulze-Lefert & Panstruga, 2011). Other genes such as enhanced disease susceptibility 1 (*EDS1*), phytoalexin-deficient 4 (*PAD4*), senescence-associated gene 101 (*SAG101*), mildew resistance locus o2 (*MLO2*), UDP-glucosyltransferase *UGT84A2/BRT1* and calcium sensor *CaM7* were identified to be involved in Arabidopsis NHR (Lipka et al., 2005; Stein et al., 2006, Nakao et al., 2011; Langenbach et al., 2013; Campe et al., 2016). In addition to these NHR genes, spatial and temporal changes in the production of stress hormones play a major role in nonhost immunity. For example, the involvement of
Salicylic acid and jasmonic acid in the expression of nonhost defense in Arabidopsis against non-adapted fungal isolates has been reported (Mellersh & Heath, 2003).

Glycine rich proteins (GRPs) belong to a protein superfamily that is characterized by the presence of a glycine-rich domain arranged in (Gly)n-X repeats. Expression of genes encoding GRPs is tissue-specific and they are often developmentally regulated or modulated by biotic and abiotic factors (Mangeon, Junqueira & Sachetto-Martins, 2010). GRPs are involved in a variety of functions in plants including cell wall structure, plant defense, pollen hydration, signal transduction, osmotic stress, cold stress, and flowering time control, development and cell elongation (Mousavi & Hotta, 2005; Mangeon, Junqueira & Sachetto-Martins, 2010). GRPs take part in plant defense responses by maintaining cell wall components and callose deposition (Ueki & Citovsky, 2002; Lin & Chen, 2014), modulating PR-1 expression (Park et al., 2001), and displaying antimicrobial activity to inhibit growth of microbes (Park et al., 2000; Egorov et al., 2005; Tavares et al., 2012). Aside from the glycine-rich domain, some GRPs carry RNA-binding domains. Arabidopsis AtGRP7 is a glycine-rich RNA binding protein that regulates callose deposition in the PAMP flg22-induced FLS2 mediated immunity (Fu et al., 2007). AtGRDP2 encodes a short glycine-rich domain protein containing a DUF1399 domain and a putative RNA recognition motif. Overexpression of AtGRDP2 resulted in higher tolerance of Arabidopsis to salinity stress (Ortega-Amaro et al., 2014).

Arabidopsis is a model plant with T-DNA mutants available for most of its genes making it suitable for studying nonhost resistance mechanisms (Alonso et al., 2003; Rhee et al., 2003). We previously reported identification of 30 P. sojae susceptible mutants named pss1 through pss30 from screening of over 3,500 ethylmethane sulfonate (EMS)-induced M2 families. The pss1 mutant was shown to be susceptible also to F. virguliforme (Sumit et al., 2012). PSS1 was genetically mapped to Chromosome 3 by bulked segregant analysis (Sumit et al., 2012). In this study, we applied SHORE mapping to identify six candidate PSS1 genes (Schneeberger et al., 2009). Analyses of T-DNA insertion mutants of the candidate PSS1 genes led to identification of the At3g59640 gene that complemented EMS-induced pss1 and two T-DNA insertion-induced pss1 mutants. We showed that PSS1 localizes to the plasma membrane. Furthermore we identified that upon stable transformation, PSS1 enhances resistance to the fungal pathogen F. virguliforme in transgenic soybean plants.
Results

The nonhost resistance PSSI gene encodes a glycine-rich protein

PSSI was mapped to a 2.75 Mb genomic region between markers SBP_20.71 and SBP_23.46 on Chromosome 3 (Sumit et al., 2012) (Fig. 1). Comparison of the sequence of the PSSI region in a bulked DNA sample generated from seven F$_{2:3}$ homozygous families for the pss1 allele with that of the Col-0 genome sequence revealed 30 point mutations or single nucleotide polymorphisms (SNPs). Nine of these mutations were non-synonymous. The pss1 mutant was generated in the Col-0 pen1-1 mutant background (Sumit et al., 2012). Three of the nine non-synonymous mutations are common to both pen1-1 and pss1 mutants and were not considered for further study. The six candidate PSSI genes, each carrying one pss1-specific non-synonymous mutation, are presented in Fig. 1 and Table 1.

To identify the candidate PSSI gene, 25 T-DNA knockout mutants for the six candidate PSSI genes (Supplemental Table S1) were evaluated for response to P. sojae infection. Of the 25 lines tested, only two T-DNA lines, SALK_090245C and SALK_148857C, showed susceptibility to P. sojae. SALK_090245C and SALK_148857C contain T-DNA insertions in Exon 1 and promoter, respectively, of the At3g59640 gene (Fig. 2A). RT-PCR failed to detect At3g59640 transcripts in either T-DNA insertion line (Fig. 2B). The pss1 and two T-DNA mutants were transformed with the At3g59640 cDNA fused to the CaMV 35S promoter. PCR amplification confirmed the stable integration of the At3g59640 transgene in the pss1, SALK_148857C, and SALK_090245C mutants transformed with the 35S:At3g59640 cDNA fusion gene (Fig. 2C). The 35S:At3g59640 transformed pss1 and T-DNA insertion pss1 mutants expressed immunity to P. sojae suggesting that At3g59640 complemented the lost immunity function of the pss1 mutants (Fig. 2D, Supplemental Fig. S1). We therefore concluded that At3g59640 is the PSSI gene.

PSSI encodes a glycine-rich protein with unknown function. Apart from the glycine-rich motif (from aa 119 to 154) (Fig. 3A), PSSI also contains a predicted transmembrane motif (aa 158 to 175) (Fig. 3B). In the EMS-induced pss1 mutant allele, a glycine residue is substituted with an aspartic acid residue at the 119th position. This mutation is located within the conserved glycine-rich domain (Fig. 3A). We hypothesized that a change in this conserved residue led to change in protein structure of PSS1 and loss of the immunity function. To investigate this, we predicted structures

PSS1 and loss of the immunity function. To investigate this, we predicted structures
of PSS1 and its mutant proteins using the I-TASSER program (Zhang, 2008). Pairwise structure alignment suggested that the pss1 mutant protein possesses low structural similarity to PSS1 (TM-score = 0.26 < threshold 0.5; Supplemental Fig. S2; Zhang & Skolnick, 2005).

Based on the arrangement of glycine-rich units, PSS1 is classified as a member of the Class VII glycine-rich proteins that carry a mixed pattern of glycine-rich repeats (Mangeon, Junqueira & Sachetto-Martins, 2010). A sequence similarity search with BLASTP identified 93 plant proteins with amino acid identity >33% to PSS1 with an E-value <1e-25. None of the 93 PSS1 homologs have been characterized. A few of them have been predicated to be major histocompatibility complex (MHC) Class II regulatory factor (XP_013615797, Brassica oleracea), autophagy-related protein 3 (JAT40787, Anthurium amnicola), and nuclear envelope protein (NP_850396, Arabidopsis thaliana). The constructed Neighbor Joining phylogenetic tree revealed that PSS1 clustered in a subclade with 10 homologs of the Brassicaceae family (Fig. 4). Alignment of these 10 PSS1 homologs and PSS1 revealed that the glycine-rich motifs and transmembrane domains were highly conserved among these glycine-rich proteins (Supplemental Fig. S3). The soybean PSS1 homologs were placed to a distinct subclade (Fig. 4). Further study is warranted to determine if anyone of the genes is
orthologous to PSS1 and governs any defense function.

**PSS1 is induced in response to *P. sojae* infection**

*PSS1* has shown no homology to any protein with known function (searched on August 16, 2017). *pss1* and knockout T-DNA insertion mutants did not show any discernible defects in general growth and root development (data not shown). To address the biological function of *PSS1*, qRT-PCR was performed. *PSS1* expression pattern was also examined by searching Arabidopsis eFP Browser (http://bar.utoronto.ca/)
which contains an extensive collection of gene expression microarray data (Winter et al., 2007). *PSS1* is induced following infection not only with *P. sojae* (Fig. 5A), but also at least 1.5 fold following infection with several pathogens including *Golovinomyces orontii*, *Hyaloperonospora arabidopsis* and *Pseudomonas syringae*, as well as in response to treatments with various elicitors including Hrpz and flg22 (Supplemental Table S2). *PSS1* expression is highest in siliques (Fig. 5B).

Co-expressed genes with the same transcriptional regulatory pathway could be functionally related, or they could be members of the same biochemical or regulatory pathway or protein complexes. We conducted initial co-expression analysis using data set from microarray platform available at ATTED-II (http://atted.jp/; Obayashi et al., 2007). Gene ontology (GO) and KEGG (Kanehisa & Goto, 2000) enrichment analysis suggested that the co-expression network is related to three biological functions; viz., autophagy, para-aminobenzoic acid metabolic process and nuclear mRNA splicing *via* spliceosome (Supplemental Fig. S4). To avoid any biases resulting in from use of single dataset, co-expression analysis was conducted also for the mRNAseq dataset available at Genevestigate (Hruz et al., 2008). GO enrichment analysis of top 25 co-expressed genes suggested that the genes putatively involved in three biological processes: (i) autophagy, (ii) protein targeting to vacuole, and (iii) vesicle-mediated transport (Fig. 5C). Utilization of different gene expression datasets is expected to yield reliable information (Ballouz, Verleyen & Gillis, 2015). To validate the out-
comes of the co-expression analyses (Fig. 5C; Supplemental Fig. S4), we conducted semi-quantitative RT-PCR of eight genes (Supplemental Table S3) selected from both mRNAseq and microarray data sets and observed that the autophagy related gene ATG8a (At4g21980) is induced upon P. sojae infection (Supplemental Table S3). ATG8a is the core gene in autophagy (Yoshimoto et al., 2004). These results indicate a possible connection of PSS1 to an autophagy-related defense mechanism (Liu et al., 2005).

**PSS1 was localized to plasma membrane**

Investigation of PSS1 using a transmembrane prediction program (http://cbs.dtu.dk/services/TMHMM/) revealed that it contains a single transmembrane domain between residues 158 to 175 (Fig. 3B). The Cell eFP browser (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi) (Winter et al., 2007) predicted that PSS1 could reside in nuclei, mitochondria, chloroplast and plasma membrane. SignalP 4.0 however did not identify any secretory signal peptides in PSS1. We ex-
pressed enhanced green fluorescent protein (eGFP) fused-PSS1 (GFP-PSS1 and PSS1-GFP) transiently in *Nicotiana benthamiana* (Fig. 6). Plasma membrane protein AtPIP2A fused to mCherry was used as a plasma membrane (PM) marker (Nelson, Cai & Nebenführ, 2007) and eGFP alone as a control. Two days after coinfiltration, the GFP fluorescence signals of PSS1-GFP were detected as sharp, thin lines at the cell periphery, and overlapped with the red fluorescence of the mCherry fused PM marker (Fig. 6). After plasmolysis, co-localization of PSS1-GFP with the PM marker was retained and detached from cell wall and obvious Hechtian strands were observed (Supplemental Fig. S5B), a characteristic of plasma membrane proteins. These re-
Results suggest that PSS1 is most likely an integral plasma membrane protein; not a cell-wall protein. GFP-PSS1 fusion protein with GFP at the N-terminus PSS1 exhibited loss of its plasma membrane localization; and instead, it showed cytoplasmic localization, similar to eGFP (Supplemental Fig. S5A). This suggests that the signal for plasma membrane localization in PSS1 is most likely located at the N-terminus.

**PSS1 transgenic soybean lines exhibited enhanced SDS resistance**

Soybean cultivar, ‘Williams 82’ was transformed with the PSS1 cDNA fused individually to three promoters: (i) Prom1, a soybean *F. virguliforme* Mont-1 infection-inducible promoter (*Glyma18g47390*; B.B. Sahu and M.K. Bhattacharyya, unpublished); (ii) Prom2, a soybean root specific promoter (*Glyma10g31210*) (http://www.oardc.ohio-state.edu/SURE/GmROOT/GmRoot.htm; Ngaki et al., 2016); and (iii) Ubi10, an Arabidopsis constitutive promoter (*At4g05320*) (Norris, Meyer & Callis, 1993) (Fig. 7A). R1 seeds were collected from R0 soybean plants grown in the greenhouse. Transgenic soybean plants carrying PSS1 did not show any obvious changes in morphology compared to the non-transgenic recipient Williams 82 cultivar. To examine the responses of transgenic plants to *F. virguliforme*, seeds were planted in a soil pre-mixed with *F. virguliforme* Mont-1 isolate and the seedlings were
grown in growth chambers. Williams 82 is a moderately susceptible line, while MN1606 is a sudden death syndrome (SDS) resistant line. Foliar SDS symptoms were recorded four weeks after planting. Approximately 1/3 to 2/3 of the selected R₃ plants were studied. Experiment was repeated two more times that showed similar results. Foliar SDS symptoms for individual plant were scored four weeks following planting. WT, transgene recipient non-transgenic Williams 82 (W82) as the SDS susceptible control; and MN1606, SDS resistant control. C, RT-PCR analysis of the transgenic R₃ plants for PSSI transcripts. Lanes 1 to 6, RT-PCR products from F. virguliforme infected roots of three independent lines carrying promoter Prom1 fused to PSSI; Lanes 7 to 12, RT-PCR products from F. virguliforme infected roots of three independent lines carrying promoter Prom2 fused to PSSI; and 13 to 18 lanes, RT-PCR products from F. virguliforme infected roots of three independent lines carrying promoter Ubi10 fused to PSSI. For each independent transgenic line two R₃ SDS resistant plants (Lanes 1 to 18) were analyzed. Lines 19 to 21, RT-PCR products from F. virguliforme infected roots of three independent R₃ progeny plants that were SDS susceptible. D, Relative biomass of F. virguliforme measured by genomic DNA quantitative PCR of the FvTox1 gene among three independent transgenic lines. Root samples were collected two weeks following infection with F. virguliforme Mont-1 isolate in a growth chamber. 206-7-1, transgenic line carrying Prom1-PSSI; 2-7-3-6, transgenic line carrying Prom2-PSSI; 227-9-1, transgenic line carrying the Ubi10-1-PSSI fusion gene; W82, Williams 82. Asterisks indicate statistical significance at p < 0.05 when compared with the biomass of F. virguliforme in Williams 82.
showed enhanced SDS resistance with disease severity ratings of < 2.0 (Fig. 7B). The nontransgenic Williams 82 line exhibited SDS severity ratings of over 4.0 among 90% of the plants and 90% of MN1606 plants showed disease ratings of < 2.0. RT-PCR analysis indicated that PSSI transcripts were present in all SDS resistant R1 progenies, but not in the SDS susceptible R1 progenies (Fig. 7C).

Foliar SDS is induced by toxins produced by F. virguliforme in infected roots (Ji, Scott & Bhattacharyya, 2006; Brar, Swaminathan & Bhattacharyya, 2011). We hypothesized that overexpressed PSSI in roots conferred enhanced root resistance against the pathogen. To test our hypothesis, seeds of three SDS resistant R2 plants, each representing one of the three transgenes: (i) Prom1-PSSI; (2) Prom2-PSSI, and (iii) Ubi10-PSSI were planted in soil mixed with F. virguliforme Mont-1 inoculum in a growth chamber. Genomic DNA qPCR was conducted for the single copy F. virguliforme FvTox1 toxin gene (Brar, Swaminathan & Bhattacharyya, 2011). The qPCR revealed that the levels of F. virguliforme growth in the roots of SDS resistant transgenic soybean plants expressing the PSSI gene under the control of Prom1, Prom2 or Ubi10, respectively, was decreased up to 85% as compared to that in Williams 82 (Fig. 7D). These results suggest that PSSI suppressed the extent of F. virguliforme’s spread in the infected roots.

To determine if transgenic SDS resistant plants showed enhanced SDS resistance under field conditions, field trials were conducted at Hinds Farms, Iowa State University in 2015 and 2016 seasons. The transgenic soybean lines carrying PSSI transgenes showed enhanced SDS resistance under field conditions (Fig. 8A). In each season, transgenic seeds were planted along with the control lines Williams 82, MN1606 or Ripley. F. virguliforme NE305S isolate grown on sorghum meal was mixed with seeds prior to sowing. In 2015, R1 seeds were sown. Three weeks after seeds germination, Basta was sprayed to transgenic lines to eliminate any azygous progenies. We sprayed Basta at early vegetative growth stages. Foliar SDS symptoms appear after flowering, and foliar symptom severity were recorded at reproductive stage (R6 stage, the stage at which weight of developing pods peaks). There was a gap of four weeks between Basta spray and SDS symptom development. Once symptoms started to appear, the SDS severity of each plant was scored. Transgenic lines, 207-4 and 207-14 expressing Prom2 driven PSSI, 227-8 and 227-9 carrying Ubi10 promoter fused PSSI and 206-7 carrying promoter Prom1 fused PSSI showed significantly enhanced SDS resistance under field conditions (p < 0.05) (Fig. 8B). Leaves of SDS resistant plants were col-
lected just after first scoring of foliar SDS severity for determining the transgene copy number (Supplemental Table S4). The harvested R₂ seeds of putative homozygous R₁ plants were grown in the 2016 field trial. In 2016, Basta herbicide was applied to eliminate any possible azygous segregants from heterozygous R₁ plants. The SDS severity index indicated that transgenic lines with promoter Prom2 and Ubi10 driven PSSI showed enhanced SDS resistance (Fig. 8C).

Figure 8. Expression of PSSI enhances SDS resistance in transgenic soybean plants under field conditions. A, Representative field plot showing SDS-resistant transgenic and Williams 82 control plants. B and C, Mean foliar SDS severity for individual transgenic lines in 2015 and 2016 field trials. Each line comprised of 12 to 66 Basta resistant R₁ or R₂ seedlings. The experiment was conducted in a randomized block design. Asterisks indicate significant reduction in foliar SDS scores between transgenic lines and non-transgenic recipient Williams 82 (W82) control at p < 0.05.
In this investigation we applied a map-based cloning approach to isolate the Arabidopsis nonhost resistance PSSI gene. The gene was mapped to a 2.75 Mb genomic region Mb. Bulked DNA sample of seven F$_{2:3}$ lines homozygous for the pss1 allele was sequenced to identify the candidate PSSI gene through SHORE mapping (Schneeberger et al., 2009). Analyses of T-DNA insertion lines and complementation analyses confirmed that PSSI encodes a glycine-rich protein. Mutations in this gene led to a loss of immunity of Arabidopsis to two soybean pathogens, P. sojae and F. virguliforme; but not to the bacterial pathogen, Pseudomonas syringae pv. glycinea that causes bacterial blight in soybean (Sumit et al., 2012).

pss1 mutant was created in the pen1-l genetic background because P. sojae can penetrate single cells of the pen1-l mutant (Sumit et al. 2012). Ecotype Col-0, on the other hand is not penetrated by P. sojae. We therefore expected to observe an epistatic effect of PEN1 on PSS1 if PSS1 was to encode a second layer of plant defense. Surprisingly, 3:1 segregating ratio was observed for the pss1 mutation (PSSI::pss1::3:1) suggesting a single gene action with no epistasis effect of PEN1 on PSS1 (Sumit et al., 2012). This observation was further supported by responses of two T-DNA insertion, lines SALK_090245C and SALK_148857C. Both mutants are susceptible to P. sojae although both carry the PEN1 allele (Fig. 2). Together with the previous study (Sumit et al., 2012), our study suggests that PSS1 may act at both pre- and post-haustorial levels, while PEN1 acts at the pre-haustorial level against this soybean pathogen.

PSSI encodes a glycine rich novel protein (GRP1). Glycine rich proteins are classified into seven classes based on the pattern of their glycine-rich domain. PSSI belongs to group VII, which carry a mixed arrangement of glycine repeats with no other conserved domains (Mangeon, Junqueira & Sachetto-Martins, 2010).

PSSI confers broad-spectrum nonhost immunity of Arabidopsis to two soybean pathogens (Sumit et al., 2012). Transgenic studies in soybean have suggested the utility of this gene in enhancing disease resistance in crop plants. We have localized the protein to plasma membrane through its transient expression in N. benthamiana (Fig. 6). The broad-spectrum disease resistance function and its putative plasma membrane location suggests a possible recognition/signaling role for the protein in the activation of host defense responses. However, we cannot rule out the possibility of other mech-
anisms including possible structural and/or chemical barriers mediated by PSS1.

GRPs are involved in multiple functions in plant defense response such as blocking virus-movement, interacting with kinases, and modulating transcription of defense genes (Park et al., 2001; Ueki & Citovsky, 2002; Ueki & Citovsky, 2005; Tao et al., 2006; Kim et al., 2007; Park et al., 2008; Nicaise et al., 2013; Kim, Kim & Hwang, 2015). PSS1 is induced by many pathogens. PAMPs such as flagellin (flg22), harpin (HrpZ), necrosis-inducing proteins (NPP) and lipopolysaccharide (LPS) can also induce its expression. Surprisingly, the bacterial PAMP, HrpZ protein is glycine-rich and triggers hypersensitive response at the infection site (Choi et al., 2013).

The subcellular localization and predicted protein structure suggest that PSS1 is an integral plasma membrane protein carrying one membrane spanning domain. Plant immunity is regulated at both transcriptional and post-transcriptional levels. pre-RNAs of target regulatory genes must be processed correctly for regulating defense responses. GRPs with RNA-binding domain have been suggested to play a role in RNA processing (Woloshen, Huang & Li, 2011). Alternate splicing has been documented as essential for expression of effector triggered immunity in tobacco (Dinesh-Kumar & Baker, 2000). Whether PSS1 has any role in RNA-splicing is yet to be investigated.

Functions of co-expressed genes showing the same transcriptional regulatory pathway could be used in predicting functions of genes. RT-PCR of seven genes that were co-expressed with PSS1 in the mRNA sequencing or transcript hybridization to microarrays studies indicated that PSS1 co-expresses with the core autophagy gene ATG8a (At4g21980) (Supplemental Table S3; Yoshimoto et al., 2004). Autophagy is a conserved intracellular trafficking and degradation process and has been shown to be linked to induction of programmed cell death or HR as part of basal plant immunity (Liu et al., 2005; Teh & Hofius, 2014). It will be important to determine if PSS1 is involved in autophagy-mediated plant immunity.

In recent years, SDS has emerged as the second most serious soybean disease after soybean cyst nematodes in USA; and in certain years it can cause yield suppression valued up to $0.7 billion (Bradley & Allen, 2014). Although first reported in 1971, the fungal pathogen, F. virguliforme causing SDS has spread to all soybean-growing states in USA and Canada (Ngaki et al., 2016). Currently the use of SDS resistant cultivars is the only option available to manage this disease. However, breeding SDS re-
sistant cultivars is not trivial since the SDS resistance is partial and governed by more than 40 QTL (Swaminathan et al., 2015). The pathogen is soil borne and remains in infected roots, where it produces toxins that cause the foliar SDS (Brar, Swaminathan & Bhattacharyya, 2011; Brar & Bhattacharyya, 2012; Pudake et al., 2013; Chang et al., 2016). Development of transgenic SDS resistant lines is a possible alternative to combat this disease. Transgenic expression of plant antibodies or interacting peptides that bind to foliar SDS inducing toxins has shown some promise in enhancing SDS resistance in soybean (Brar and Bhattacharyya, 2012; Wang, Swaminathan & Bhattacharyya, 2015; B. Wang and M.K. Bhattacharyya, unpublished).

Our study suggests that nonhost disease resistance governed by PSSI can enhance SDS resistance in transgenic soybean plants by restricting the spread of fungal growth in the infected roots (Fig. 7D). It’s very unlikely that the enhanced SDS resistance in the transgenic lines was induced by Basta spray, as was observed in an earlier study conducted in transgenic rice (Ahn, 2008), because of the following reasons. In the growth chamber assays, we never sprayed Basta (Fig. 7). Secondly, although we sprayed Basta in both 2015 and 2016 growing seasons in the field grown soybean plants, not all transgenic soybean lines were SDS resistant; some are as susceptible as the non-transgenic Williams 82 plants (Fig. 8).

The PSSI encoded resistance mechanism could complement the natural SDS resistance mechanisms and be suitable in breeding SDS resistant soybean lines. Considering the wide-spread cultivation of transgenic soybean world-wide; e.g., about 94% of the soybean crop grown in the USA and 81% world-wide are transgenic (Perry et al., 2016), development of SDS resistant transgenic plants could be a good alternative to facilitate soybean breeding programs for SDS resistance.

In summary, PSSI encodes a novel unknown mechanism to confer nonhost resistance of Arabidopsis against two important soybean pathogens, P. sojae and F. virguliforme. Its’ plasma membrane localization and induction in response to infection by multiple pathogens and treatment with PAMPs suggest its possible regulatory role in plant defenses. It is possible that PSSI may confer its plant immunity function through autophagy. The transgenic study of PSSI revealed that the transfer of non-host resistance genes could be an important strategy in engineering disease resistance in crop plants.
Materials and Methods

Plants and pathogens

Arabidopsis plants including the wild-type ecotypes Col-0 and Niederzenz as well as mutants were grown on LC1 soil (Sun Gro Horticulture, Bellevue, WA, USA) in growth chambers at 21°C and 60% humidity with a dark/light cycle of 8/16 hours and a light intensity of 100 µmol m⁻² sec⁻¹. Soybean variety ‘Williams 82’ and transgenic lines were grown on Metro Mix 910 (Sun Gro Horticulture, Agawam, MA, USA) at 23°C, 60% humidity with a dark/light cycle of 8/16 hours and a light intensity of 300 µmol m⁻² sec⁻¹ in growth chambers. The P. sojae NW strain was maintained on V8 agar plates, and F. virguliforme isolates on PDA plates.

Nonhost resistant gene cloning

The PSSI gene was previously mapped to the lower arm of Chromosome 3 between marker SBP_20.71 and SBP_23.46 by conducting bulked segregation analysis in a segregating population developed from a cross between the pssl mutant x Niederzenz (Sumit et al., 2012). Subsequently, genomic DNA of seven homozygous susceptible pssl/pssl F₂:₃ families were extracted using the CTAB method (Murray & Thompson, 1980) and bulked for sequencing on Illumina HiSeq 2500 platform at the Iowa State University DNA Facility. The short sequencing reads were assembled into contigs, which were aligned to the reference Col-0 sequence to identify mutations in the 2.75 Mb pssl region using the SHORE program (Schneeberger et al., 2009). Because the pss mutants were developed in the pen1-1 mutant, any mutations originating from pen1-1 were not considered for further analysis.

Homozygous T-DNA knockout lines for the candidate PSSI genes carrying nonsynonymous mutations were obtained from the Arabidopsis Biological Resource Center (ABRC) located at the Ohio State University and individual T-DNA mutant line was verified by PCR amplification (Supplemental Table S1). Leaves of three-week old T-DNA insertion mutant lines, pssl, pen1-1 and Col-0 plants were inoculated with 20 µl of P. sojae NW zoospores (5×10⁵ ml⁻¹) as previously described (Sumit et al., 2012). Symptoms were scored three and four days after inoculation.

To complement pssl and T–DNA insertion mutant lines, the nonhost resistant cDNA was amplified by RT-PCR from the Col-0 transcripts and inserted into the binary vector pTF101.1 under the control of CaMV 35S promoter. Sequencing was per-
formed to confirm identity of the \textit{PSSI} gene. The resulting construct was transformed into \textit{Agrobacterium tumefaciens} strain EHA101 by following the freeze-thaw method. \textit{pssi} and T-DNA insertion mutant lines were transformed by conducting floral dip of the mutants with the \textit{A. tumefaciens} EHA101 isolate carrying the candidate \textit{PSSI} gene (Weigel & Glazebrook, 2006). T\textsubscript{1} and T\textsubscript{2} progenies were screened for Basta resistance by spraying with Liberty (80 µg/ml) herbicide. T\textsubscript{3} plants along with controls Col-0, \textit{pssi} mutant and T-DNA insertion lines were inoculated with \textit{P. sojae} spores to examine their disease phenotypes.

\textbf{Quantitative reverse transcriptase PCR (q-RT-PCR)}

For quantitative reverse transcriptase (RT)-PCR (q-RT-PCR) analyses of the \textit{PSSI} gene, three leaves of three-week old \textit{pssi} mutant plants were inoculated with 20 µl of \textit{P. sojae} NW zoospores (5×10\textsuperscript{5}/ml) or water. Leaf samples were collected 6, 12, 24, 36, 48, 72 and 96 h after inoculation in three independent experiments. Total RNA was extracted using the SV Total RNA isolation kit (Promega, Madison, WI, USA). The isolated RNAs were reverse transcribed into cDNA using the superscript first strand synthesis system (Thermo Fisher Scientific, Waltham, MA, USA). Transcript amounts of the \textit{PSSI} and actin gene were examined by conducting q-RT-PCR with \textit{PSSI} and \textit{Actin}-specific primers (Supplemental Table S5). q-RT-PCR was conducted using SYBR\textsuperscript{®} Green master mixes (Thermo Fisher Scientific, Waltham, MA, USA) by following the manufacturer’s instruction kit manual. For the study of tissue specific expression of \textit{PSSI} in various tissues including stem, roots, flowers, leaves and siliques, RNA extraction was conducted as described earlier for leaves. The induced fold changes in the \textit{PSSI} expression were calculated against the mock control.

To quantify the pathogen biomass in infected soybean roots, a genomic DNA-PCR was conducted for the DNA isolated from the transgenic and non-transgenic Williams 82 soybean plants infected with \textit{F. virguliforme} (Mont-1). DNA was diluted to 20 ng/µl for qPCR to quantify the single copy \textit{F. virguliforme FvTox1} gene (Brar, Swaminathan & Bhattacharyya, 2011) as a measure of fungal biomass. The single copy soybean gene, \textit{Glyma.05G014200} was used as internal control. qPCR run in iQ5 Biorad instrument using the SYBR green protocol. The primers used for qPCR of \textit{Glyma.05G014200} were evaluated earlier (Ngaki et al., 2016) and presented in Supplemental Table S5. For qPCR of \textit{FvTox1}, primers previously developed for quantifi-
cation of *FvTox1* and *F. virguliforme* biomass were used (Mbofung et al., 2011; Supplemental Table S5).

**Reverse transcriptase PCR (RT-PCR)**

To investigate the expression of *PSS1* and identified genes that are co-expressed with *PSS1*, leaves of Col-0, and *pss1* were inoculated with 20 µl *P. sojae* NW isolate zoospore suspension (10^5 zoospores/ml). Inoculated leaves were harvested in a time course (0, 6, 12 and 24 h post-inoculation) and the RNAs were isolated and subjected to RT-PCR using primers specific for each gene (Supplemental Table S5). Arabidopsis *Actin* gene was used as an internal control. The intensity of PCR bands of individual samples were quantified by using ImageJ (http://rsb.info.nih.gov/ij/index.html).

We followed the procedure outlined in the ImageJ document to collect the pixelated data (https://imagej.nih.gov/ij/docs/user-guide-A4booklet.pdf, p. 129). The data from three independent experiments were analyzed for statistically significant differences for *P. sojae* infected and uninfected water control leaf tissues for eight genes including *PSS1* using the open source R program (Supplemental Table S3).

**Subcellular localization of PSS1**

The *PSS1* gene was fused at the N- and C-termini of GFP and cloned in pISUAgron5 vector (S. Li, N.N. Narayanan and M.K. Bhattacharyya, unpublished), in which eGFP is already fused to the CaMV 35S promoter. pISUAgron5 vector was used as the GFP control. Plasma membrane marker (PM), Arabidopsis PIP2A fused to the mCherry tag was obtained from ABRC (Nelson, Cai & Nebenführ, 2007). For Agrobacterium-mediated transient transformation, individual *A. tumefaciens* isolates containing each of the two GFP fusion constructs or control GFP construct was co-infiltrated with PM marker into leaves of four-week-old *Nicotiana benthamiana* plants (Shamloul et al., 2014). Two days following infiltration, small leaf pieces were mounted in either water or 1 M NaCl. Samples were observed under a 20X oil immersion lens mounted to a Leica SP5 X MP confocal/multiphoton inverted microscope. To monitor GFP fluorescence, a 488 nm argon laser and PMT detector with emission bandwidth set to 495-550 nm was used. To monitor the mCherry signal, a HeNe 561 laser (561 nm) and a third PMT detector (587-610 nm) was used (Schweiger & Schwenkert, 2014).
Generation of transgenic soybean lines

The gene \textit{PSS1} was first cloned into vector pGEM®-T (Promega, Madison, WI, USA) and sequenced to confirm its identity. The gene was then released from the pGEM®-T vector and cloned in the modified binary pTF102 vectors carrying one of three promoters; Prom1, Prom2 and Ubi10, respectively. Prom1 is a soybean infection inducible promoter (\textit{Glyma18g47390}) (B.B. Sahu and M.K. Bhattacharyya, unpublished). Prom2 is a soybean root specific promoter (\textit{Glyma10g31210}) (http://www.oardc.ohio-state.edu/SURE/GmROOT/GmRoot.htm; Ngaki et al., 2016). Ubi10 promoter was isolated from the Arabidopsis \textit{At4g05320} gene (Norris, Meyer & Callis, 1993). The resulting three constructs were transformed into \textit{A. tumefaciens} strain EHA101 for generating stable transformants in the soybean Williams 82 cultivar at the Plant Transformation Facility, Iowa State University (Paz et al., 2004). Basta (Glufosinate) resistant R\textsubscript{0} plants were tested for incorporation of the \textit{bar} gene by PCR. For each \textit{PSS1} construct, at least three transgenic events were generated. Basta resistant R\textsubscript{0} plants were grown in greenhouse to maturity for harvesting R\textsubscript{1} seeds.

Evaluation of \textit{PSS1} transgenic lines in growth chamber and under field conditions for SDS resistance

R\textsubscript{1} progeny derived from self-pollinated R\textsubscript{0} plants were investigated for possible enhanced SDS resistance under growth chamber conditions. The \textit{F. virguliforme} inocula were prepared and colony-forming units of the inocula were determined as described previously (Li, Hartman & Chen, 2009). To prepare the inoculum, 500 g sorghum grains were first soaked in distilled water overnight and then washed five times to remove sorghum seeds and debris that were floated. The excess water was drained, and grains were autoclaved for 40 min at 121°C. Each of the flasks containing sterilized sorghum grains was inoculated with \textit{F. virguliforme} isolate Mont-1 by transferring ten 20-mm-diameter plugs from 1/3 PDA plates containing two-week old \textit{F. virguliforme} Mont-1 culture. Flasks were then incubated at room light and temperature and shaken gently by hand every other day for two weeks to ensure uniform fungal growth. After one month, the sorghum grains, infested with the fungus, were dried for 24 h under a fume hood. Infested kernels were then stored at 4 °C until further use typically no longer than 3 months.

For growth chamber assays, a 2:1 mixture of sand and soil was mixed with the in-
ocula at a ratio of 19:1 :: soil mix: inocula and placed in 237 ml Styrofoam cups for
sowing three seeds of each genotype. Fifteen seeds of each soybean line were evalu-
ated in five Styrofoam cups. Plants were grown in a growth chamber at 22-23°C and
with light intensity of 300 μmol photons m^-2 sec^{-1} (Luckew et al., 2013). Foliar symp-
toms were scored three and four weeks following planting (Hartman et al., 1997).

R1 and R2 seeds were evaluated in field trials in two consecutive seasons; from
June 11 to October 30, 2015 and from June 1 to October 15, 2016 at Hinds Research
Farm, Iowa State University, located four miles north of Ames, IA. Field trials were
carried out using a completely randomized block design with two replications in 2015
trial and three replications in 2016 trial. Twenty-four seeds of each genotype were
mixed with approximately 5 ml inocula of the F. virguliforme NE305S isolate and
sown using a hand-push planter. At the unifoliate stage, transgenic lines were sprayed
with glufosinate herbicide (250 mg/L) mixed with 0.1% Tween 20. The spray was
repeated three days later. The field was heavily irrigated to generate favorable condi-
tions for SDS symptom development. In 2016 trial only homozygous transgenic lines
showing 100% herbicide resistance were scored for foliar SDS. Individual plants were
scored for foliar SDS symptoms using a scale of 1 to 7 (modified from Hartman et al.,
1997) with 1, no symptoms, 2, slight symptom development, with mottling and mosaic
on leaves (1 to 20% foliage affected), 3, moderate symptom development, with
interveinal chlorosis and necrosis on foliage (21 to 50% foliage affected), 4, heavy
symptom development, with interveinal chlorosis and necrosis (51 to 80% foliage af-
fected), 5, severe interveinal chlorosis and necrosis (81 to 100% foliage affected), 6,
whole leaf necrosis, and 7, death of plants.

Molecular characterizations of transgenic plants

To verify gene expression in transgenic lines or Arabidopsis lines, total RNA was
extracted using the SV Total RNA isolation system (Promega, Madison, WI, USA),
following the manufacturer’s instructions, and quantified using a NanoDrop ND-1000
spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The isolated RNAs
were reverse transcribed into cDNAs using the Superscript first strand synthesis sys-
tem for RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). Semi-quantitative
RT-PCR was conducted for the PSS1 gene or co-expressed genes along with ELF1B
as the internal control.

Transgene copy numbers were determined by qPCR. Young leaves of transgenic
plants were collected in the field and genomic DNA was extracted at the Iowa State University DNA Facility using Autogen Autogenprep 740 DNA extraction robot (AutoGen, Holliston, MA, USA). DNA amount of each sample was measured by nanodrop spectrophotometer, then was diluted to 20 ng per μl for qPCR reaction. qPCR was conducted on a Biomark HD system using the 192.24 Taqman CNV protocol (Fluidigm, South San Francisco, CA, USA). Two Taqman assays were designed (Supplemental Table S5), the bar gene (target) and the reference gene (an endogenous single copy gene, Glyma.05G014200). Reporter/quencher dyes used were FAM/MGB-NFQ for bar and VIC/TAMRA for the reference gene. Data were analyzed using a Biomark HD data collection software and from the analyzed data the copy number for the bar gene was calculated.

**Statistical analysis**

All data are presented as mean ± standard error (S.E.) from at least three biological replications. The statistical significance of the difference was determined by conducting Student’s t-test. Differences between treatments were considered significant when \( p < 0.05 \) in a two-tailed test. The statistical analysis for significant differences was also conducted using the open source R program.

**Bioinformatics analyses**

The conserved motifs in PSS1 protein were predicated using MyHits program (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The prediction of signal peptide was conducted using SignalP 4.0 (Petersen et al., 2011). The transmembrane domain was predicted at the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Genes co-expressed with PSS1 were identified from the ATTED-II database (http://atted.jp/) and Genevestigator (Hruz et al., 2008) using microarray and mRNAseq data set, respectively. The phylogenetic tree of PSS1 homologs was generated by the Neighbor-Joining method with 1,000 bootstrap replications using MEGA 7 program (Kumar, Stecheer & Tamura, 2016). Protein structure prediction of PSS1 and its mutant and their pairwise structure alignment were accomplished by using I-TASSER and TM-align programs, respectively (http://zhanglab.ccmb.med.umich.edu/).
The following supplementary materials are available.

**Supplemental Figure S1.** Complementation analysis of the pss1 mutant with the CaMV 35S promoter fused PSS1 cDNA.

**Supplemental Figure S2.** Structure comparison of PSS1 and its mutant pss1 protein.

**Supplemental Figure S3.** Alignment of PSS1 homologs.

**Supplemental Figure S4.** Coexpression gene analysis based on microarray dataset.

**Supplemental Figure S5.** Subcellular localization of N- or C- terminal GFP tagged PSS1

**Supplemental Table S1.** T-DNA insertional lines used for identification of the candidate PSS1 gene.

**Supplemental Table S2.** Top 15 biotic stresses that induce expression of PSS1.

**Supplemental Table S3.** Induction of the gene (At4g21980) encoding an autophagy-related protein 8A co-expressed with PSS1 in pss1.

**Supplemental Table S4.** Pss1 transgene copy number among the R1 plants.

**Supplemental Table S5.** Primers used in this study.

Table 1. Six candidate PSS1 genes carrying non-synonymous mutations between the
NGA707 and SBP_22.95 markers mapped to Arabidopsis Chromosome 3.

<table>
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<td>AT3G59640</td>
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<td>G-A</td>
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Figure legends

**Figure 1.** Candidate nonhost resistance *PSS1* genes. The six putative nonhost resistant genes are shown in a 1.2 Mb genomic region flanked by NGA707 and SBP_22.95 markers mapped to Chromosome 3. The arrow heads indicate the orientation of six candidate *PSS1* genes on the Arabidopsis Col-0 genome sequence.

**Figure 2.** Identification of *PSS1* through mutant and complementation analyses. A, Analyses of T-DNA mutants in the *At3g59640* gene. Locations of the T-DNA insertions in the *At3g59640* gene between the two *P. sojae* susceptible T-DNA mutants, i.e. SALK_090245C and 148857C, are shown by arrows. A red asterisk shows the nonsynonymous transition G to A mutation in Exon II, which results in substitution of Gly (G) to Asp (D) at the 119th position in the pss1 mutant protein. Black boxes repre-
sent three exons and the thin lines connecting exons represent introns. The promoter is shown with a dashed line. B, Molecular analyses of pss1 mutants. RT-PCRs confirm the absence of PSSI transcripts in two T-DNA mutants shown in the left two panels. The ethyl methanesulfonate (EMS)-induced pss1 mutant is confirmed by AciI enzyme digestion of the PCR products of genomic DNA from pss1 and Col-0. Note that the transition mutation led to loss of the restriction site in the pss1 mutant. C, Molecular analyses of the PSSI cDNA transformed pss1 mutants. Electrophoresis of PCR amplified PSSI gene sequences from the EMS-induced pss1 mutant, SALK_148857C, and SALK_090245C T-DNA mutants transformed with the 35S:PSSI cDNA gene. D, PSSI complemented the pss1 mutants. Phenotypes of the pss1 and two T-DNA-insertion mutants in the At3g59640 gene and their respective complemented transgenic plants 3 days following P. sojae infection are shown.

Figure 3. PSSI encodes a glycine-rich protein containing a putative glycine-rich motif and a transmembrane domain. A, The schematic diagram of the PSSI1 protein. A red asterisk indicates the substitution of the 119th Gly with the Asp residue and two grey boxes represent a glycine-rich motif (119 to 154 aa residues) and a transmembrane domain (158 to 175 aa residues). B, Predicted transmembrane helix between amino acid residues 158 to 175 of PSSI (>90% certainty).

Figure 4. Phylogenetic tree of the PSSI homologs. Ninety-three PSSI homologs were used to construct the phylogenetic tree. PSSI is denoted with a red rectangle. The subclade containing PSSI is shown in blue, whereas the subclade with soybean PSSI homologs is presented in green. The percent identities between PSSI and soybean homologs are ≤38%.

Figure 5. Expression of PSSI and genes that show expression patterns similar to PSSI. A, Expression of PSSI following P. sojae infection. Quantitative reverse transcriptase-PCR (qRT-PCR) of PSSI was conducted following inoculation of Arabidopsis leaves with P. sojae in three independent experiments. The fold change values are relative to mock control. PSSI expression levels with asterisks were significantly induced ($p < 0.05$) when compared to 0 h control. B, Expression patterns of PSSI among various Arabidopsis tissues. qRT-PCR expression data of PSSI were collected among
Arabidopsis organs in three independent experiments. Expression comparison was against the levels in leaves ($p < 0.05$). Data in A and B are from three biological replicates and data were standardized against the transcript levels of the Actin gene. C, Gene ontology enrichment (Biological Process) of PSSI coexpression genes. The co-expression genes analysis was based on mRNA-seq data set using software Genevestigator (Hruz et al., 2008).

Figure 6. PSS1 is localized to plasma membrane. A, PSS1-GFP fusion and mCherry tagged plasma membrane (PM) marker Arabidopsis PIP2A co-localized to plasma membrane of the epidermal cells of Nicotiana benthamiana. B, the co-localized PSS1-GFP and PIP2A-mCherry fluorescent proteins remain as a complex following plasmolysis with 1 M NaCl. C, Control GFP fluorescent protein was localized to cytoplasm. D, Plasmolysis of the cell co-expressing the GFP and PIP2A-mCherry proteins. White arrows indicate Hechtian strands (see Supplemental Fig. S5 for details). Scale bars: 50 μm in PSS1-GFP panels; 25 μm in GFP alone panels.

Figure 7. Expression of PSSI enhances SDS resistance in transgenic soybean plants under growth chamber conditions. A, schematic depiction of promoter-PSSI fusion genes along with the CaMV 35S promoter fused bar gene in three binary plasmids used to generate transgenic soybean plants. B, Responses of the transgenic lines to root-infection with F. virguliforme Mont-1 in growth chambers. Plants with foliar SDS scores soybean plants. B, Responses of the transgenic lines to root-infection with F. virguliforme Mont-1 in growth chambers. Plants with foliar SDS scores $\leq 2$ were considered resistant and with $>2$ scores as susceptible. Percent resistant and susceptible R1 progenies are presented for each of the PSSI transgenes generated by fusing the PSSI gene to Prom1, Prom2 and Ubi10 promoters. For each transgenic event, 15 R1 plants were studied. Experiment was repeated two more times that showed similar results. Foliar SDS symptoms for individual plant were scored four weeks following planting. WT, transgene recipient non-transgenic Williams 82 (W82) as the SDS susceptible control; and MN1606, SDS resistant control. C, RT-PCR analysis of the transgenic R1 plants for PSSI transcripts. Lanes 1 to 6, RT-PCR products from F. virguliforme infected roots of three independent lines carrying promoter Prom1 fused to PSSI; Lanes 7 to12, RT-PCR products from F. virguliforme infected roots of three independent lines carrying promoter Prom2 fused to PSSI; and 13 to18 lanes, RT-
PCR products from *F. virguliforme* infected roots of three independent lines carrying promoter Ubi10 fused to *PSS1*. For each independent transgenic line two R1 SDS resistant plants (Lanes 1 to 18) were analyzed. Lines 19 to 21, RT-PCR products from *F. virguliforme* infected roots of three independent R1 progeny plants that were SDS susceptible. D, Relative biomasses of *F. virguliforme* measured by genomic DNA quantitative PCR of the *FvTox1* gene among three independent transgenic lines. Root samples were collected two weeks following infection with *F. virguliforme* Mont-1 isolate in a growth chamber. 206-7-1, transgenic line carrying *Prom1-PSS1*; 2-7-3-6, transgenic line carrying *Prom21-PSS1*; 227-9-1, transgenic line carrying the *Ubi10-1-PSS1* fusion gene; W82, Williams 82. Asterisks indicate statistical significance at $p < 0.05$ when compared with the biomass of *F. virguliforme* in Williams 82.

**Figure 8.** Expression of *PSS1* enhances SDS resistance in transgenic soybean plants under field conditions. A, Representative field plot showing SDS-resistant transgenic and Williams 82 control plants. B and C, Mean foliar SDS severity for individual transgenic lines in 2015 and 2016 field trials. Each line comprised of 12 to 66 Basta resistant R1 or R2 seedlings. The experiment was conducted in a randomized block design. Asterisks indicate significant reduction in foliar SDS scores between transgenic lines and non-transgenic recipient Williams 82 (W82) control at $p < 0.05$. 


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