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Arabidopsis non-host resistance *PSS30* gene enhances broad-spectrum disease resistance in the soybean cultivar Williams 82

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SUMMARY

Non-host resistance (NHR), which protects all members of a plant species from non-adapted or non-host plant pathogens, is the most common form of plant immunity. NHR provides the most durable and robust form of broad-spectrum immunity against non-adaptive pathogens pathogenic to other crop species. In a mutant screen for loss of Arabidopsis (*Arabidopsis thaliana*) NHR against the soybean (*Glycine max* (L.) Merr.) pathogen *Phytophthora sojae,* the *Phytophthora sojae-susceptible 30* (*pss30*) mutant was identified. The *pss30* mutant is also susceptible to the soybean pathogen *Fusarium virguliforme*. *PSS30* encodes a folate transporter, AtFOLT1, which was previously localized to chloroplasts and implicated in the transport of folate from the cytosol to plastids. We show that two Arabidopsis folate biosynthesis mutants with reduced folate levels exhibit a loss of non-host immunity against *P. sojae*. As compared to the wild-type Col-0 ecotype, the steady-state folate levels are reduced in the *pss1, atfolt1* and two folate biosynthesis mutants, suggesting that folate is required for non-host immunity. Overexpression of *AtFOLT1* enhances immunity of transgenic soybean lines against two serious soybean pathogens, the fungal pathogen *F. virguliforme* and the soybean cyst nematode (SCN) *Heterodera glycines*. Transgenic lines showing enhanced SCN resistance also showed increased levels of folate accumulation. This study thus suggests that folate contributes to non-host plant immunity and that overexpression of a non-host resistance gene could be a suitable strategy for generating broad-spectrum disease resistance in crop plants.

Keywords: Arabidopsis non-host resistance, *Phytophthora sojae, Fusarium virguliforme,* folate biosynthesis, folate transporter, soybean sudden death syndrome, soybean cyst nematode, *Heterodera glycines*.

INTRODUCTION

Plant immunity to pathogens is highly complex and manifested at many levels. Broad-spectrum disease resistance is elicited by pathogen-associated molecular patterns (PAMPs) and is termed PAMP-triggered immunity (PTI) or basal host resistance (Jones and Dangl, 2006). Phytopathogen effector proteins suppress PTI to cause susceptibility. This is known as effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). To defend against such invading pathogens, disease resistance (*R*) genes encoding receptors that recognize the effectors for ETS have evolved to signal the activation of effector-triggered immunity (ETI). Most *R* genes encode receptors that contain a nucleotide-binding site and leucine-rich repeat regions (NBS-LRR proteins) (Jones and Dangl, 2006). While PTI provides horizontal or broad-spectrum resistance against many pathogens, ETI provides vertical resistance against a specific set of isolates of a pathogenic species that can overcome non-host resistance (NHR).

The most common form of plant immunity is termed NHR, which protects all members of a plant species from

non-adapted or non-host plant pathogens. It provides the most durable and robust form of broad-spectrum immunity to almost all organisms that cause diseases in other plant or crop species. NHR is classified into two types. Type I is the predominant form and does not produce any visible disease symptoms, whereas Type II exhibits a hypersensitive reaction characterized by (i) rapid host cell death, (ii) accumulation of reactive oxygen species (ROS) and antimicrobial compounds and (iii) modification of cell walls at infection sites (Ayliffe and Sorensen, 2019; Dangl and Jones, 2001; Lee et al., 2017a,b; Lipka et al., 2005). Previously characterized Type I NHR genes in Arabidopsis include (i) *PEN1*, *PEN2* and *PEN3*, which prevent penetration by the non-adapted barley (*Hordeum vulgare*) fungal pathogen *Blumeria gramini* f. sp. *hordei* (Collins et al., 2003; Fuchs et al., 2016; Lipka et al., 2005; Stein et al., 2006), (ii) *NON-HOST1* (*NHO1*), which encodes a glycerol kinase and confers immunity against non-adapted and adapted bacterial pathovars of *Pseudomonas syringae* (Kang et al., 2003; Lu et al., 2001) and (iii) *PSS1*, which encodes a glycine-rich protein that confers immunity against the non-adaptive oomycete pathogen *Phytophthora sojae* and the fungal pathogen *Fusarium virguliforme* (Wang et al., 2018). Type II NHR genes include Arabidopsis enhanced disease susceptibility 1 (*EDS1*), *phytoalexin-deficient 4* (*PAD4*), *senescence-associated gene 101* (*SAG101*), *phospholipid:sterol acyltransferase 1* (*PSAT1*) and *enhanced disease resistance 1* (*EDR1*), all of which are involved in post-invasion immunity (Geissler et al., 2015; Kopischke et al., 2013; Lipka et al., 2005).

Recent studies have indicated that the molecular basis of NHR is highly complex. A large number of factors are involved in the expression of durable and broad-spectrum NHR. For example, the plant hormones salicylic acid and jasmonic acid, which regulate active host defenses, have also been shown to be involved in the expression of NHR against cowpea (*Vigna unguiculata*) rust, *Uromyces vignae* and Asian soybean rust (*Phakopsora pachyrhizi*) (Glazebrook, 2005; Lee et al., 2016; Loehrer et al., 2008; Mellersh and Heath, 2001). It has been demonstrated that Arabidopsis heterotrimeric G-proteins and nucleolar GTP-binding protein 1 (NOG1) with GTPase activity are involved in both host and non-host resistance (Lee et al., 2013, 2017a,b). Translation of a set of genes regulated by either of the RPL12 and RPL19 ribosomal proteins has been implicated in NHR against bacterial pathogens (Nagaraj et al., 2016). It has also been demonstrated that stomatal closure is regulated by an Arabidopsis AAA^{+}-ATPase encoded by *AtGCN4*. Silencing of *AtGCN4* enhances susceptibility of Arabidopsis to both host and non-host pathogens (Kaundal et al., 2017). An NADPH-dependent thioredoxin reductase C contributes to NHR against bacterial pathogens by regulating chloroplast-generated ROS in Arabidopsis (Ishiga et al., 2016). Two proline metabolism-related genes, *ProDH1* and *ProDH2*, are involved in NHR in Arabidopsis against the non-host pathogen *Pseudomonas syringae* (Fonseca and Mysore, 2019; Senthil-Kumar and Mysore, 2012). Recently, it has also been demonstrated that two chloroplast-localized proteins, AtNHR2A and AtNHR2B, are involved in regulating the callose deposition required for non-host immunity in Arabidopsis (Singh et al., 2018).

Soybean (*Glycine max* L. Merr) is one of the most valuable crops worldwide. Soybeans have high protein (approximately 40%) and oil (approximately 20%) content. In addition to human nutrition, soybean is a major protein source of both fish and animal feed. Unfortunately, soybean is susceptible to several pathogenic organisms that suppress its yield, valued over 5 billion dollars annually just in the United States (Allen et al., 2017). To determine if NHR can be utilized to generate broad-spectrum disease resistance in soybean, we conducted a genetic screen for Arabidopsis mutants that are susceptible to two soybean pathogens: (i) the oomycete pathogen *Phytophthora sojae* and (ii) the fungal pathogen *Fusarium virguliforme*. We identified 30 ***P**hytophthora **s**ojae*-**s**usceptible (*pss*) mutants *pss1* through *pss30*, of which 14 were also susceptible to *F. virguliforme* (Sumit et al., 2012; Wang et al., 2018). Here we report that the Arabidopsis NHR *PSS30* gene encodes the folate transporter AtFOLT1, which was previously localized to the chloroplast envelope and implicated in transport of folate from the cytosol to plastids (Bedhomme et al., 2005). Based on mutant and transgenic studies we conclude that folate contributes to NHR. Overexpression of *PSS30* (*AtFOLT1*) in soybean enhances broad-spectrum resistance against its two serious pathogens, the fungal pathogen *F. virguliforme* and the nematode *Heterodera glycines*.

RESULTS

The Arabidopsis non-host resistance *PSS30* gene encodes a folate transporter

The ethyl methanesulfonate (EMS)-induced Arabidopsis *pss30* mutant is susceptible to the non-adapted soybean pathogens *P. sojae* and *F. virguliforme* (Figure 1a,b; Figure S1) and was used for map-based cloning of the *PSS30* gene. The *pss30* mutant was crossed to Niederzenz (Nd-0) to generate a segregating population for mapping the *PSS30* gene. The segregation pattern among the $F_{2:3}$ families suggested that *PSS30* is a single gene (Table S1). Bulked segregation analysis was conducted for molecular mapping of the *PSS30* gene (Figure S2) (Michelmore et al., 1991). Five $F_{2:3}$ families susceptible to *P. sojae* were bulked and sequenced using the Illumina HiSeq 3000 sequencing platform. SHORE mapping was conducted to identify the non-synonymous mutations that mapped to the *PSS30* region (Ossowski et al., 2008; Schneeberger et al., 2009). Three genes carrying non-synonymous mutations that

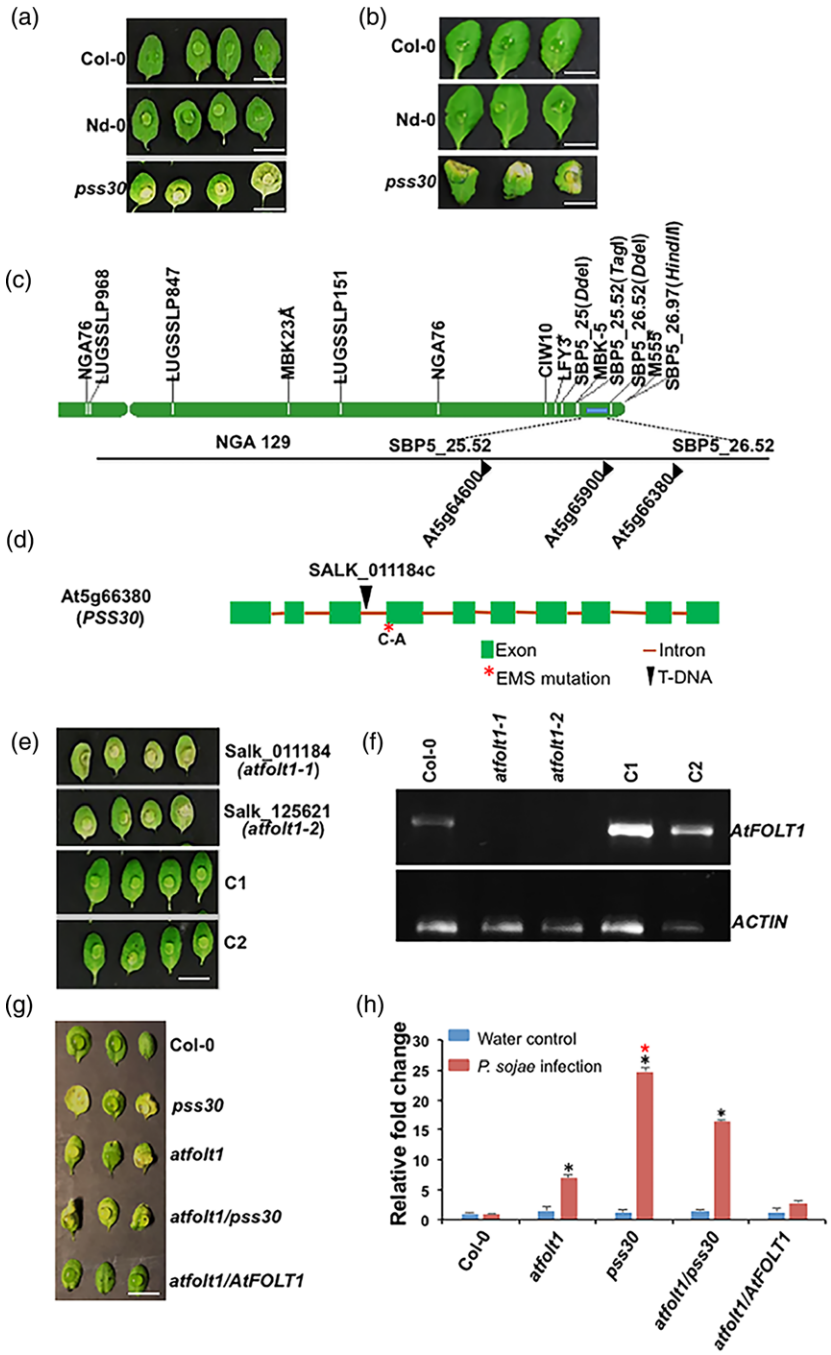

Figure 1. Map-based cloning of the Arabidopsis non-host *PSS30* gene.
(a) Phenotype of the ethyl methanesulfonate (EMS)-induced Arabidopsis *pss30* mutant following infection with *P. sojae* zoospores. (b) Phenotype of the Arabidopsis *pss30* mutant following infection with *F. virguliforme* conidiospores. (c) Three candidate *PSS30* genes identified from a 998-kb *PSS30* region flanked by SBP_25.52 and SBP_26.52 markers (Figure 2) on the south arm of Chromosome 5. (d) Structure of the candidate *PSS30* (*At5g66380*) gene showing the location of the T-DNA insertion (inverted black triangle) and the point mutation (C-to-A transversion mutation, red asterisk) (Table 1). (e) Phenotype of T-DNA knockout mutants (Salk_011184 [*atfolt1-1*] and Salk_125621 [*atfolt1-2*]) and their respective complemented lines, C1 (*atfolt1-1*:35S-*AtFOLT1*) and C2 (*atfolt1-2*:35S-*AtFOLT1*), transformed with *AtFOLT1* (*At5g66380*) following *P. sojae* inoculation. Symptoms were evaluated at 48 h post-inoculation. (f) Reverse transcriptase-PCR confirming the expression of *AtFOLT1* in the complemented lines, but not in the two T-DNA insertion knockout mutants. (g) Phenotypes of the *pss30* and *atfolt1-1* mutants, an F_1 and the complemented *atfolt1-1* mutant. (h) Relative fold changes of the *P. sojae* actin gene *ActA* among the genotypes. The Arabidopsis *AtUB5* gene was used as the internal control gene in the quantitative PCR assays. Leaf samples were collected at 3 days following *P. sojae* inoculation. $^*P < 0.05$ compared with Col-0. Bars in (a) and (b) represent 5 mm.

Table 1 Candidate *PSS30* genes carrying non-synonymous mutations

Position	Wild-type	Mutant	Mutation	Gene ID	Protein ID
25 827 439	G	A	Gly to Arg	*At5g64600*	*O*-fucosyltransferase family protein
26 358 667	G	A	Glu to Lys	*At5g65900*	Dead(d/h) box RNA helicase family protein
26 515 178	G	T	Leu to Iso	*At5g66380*[a]	Folate transporter 1 in chloroplast envelope

[a]*At5g66380* is located on the negative strand.

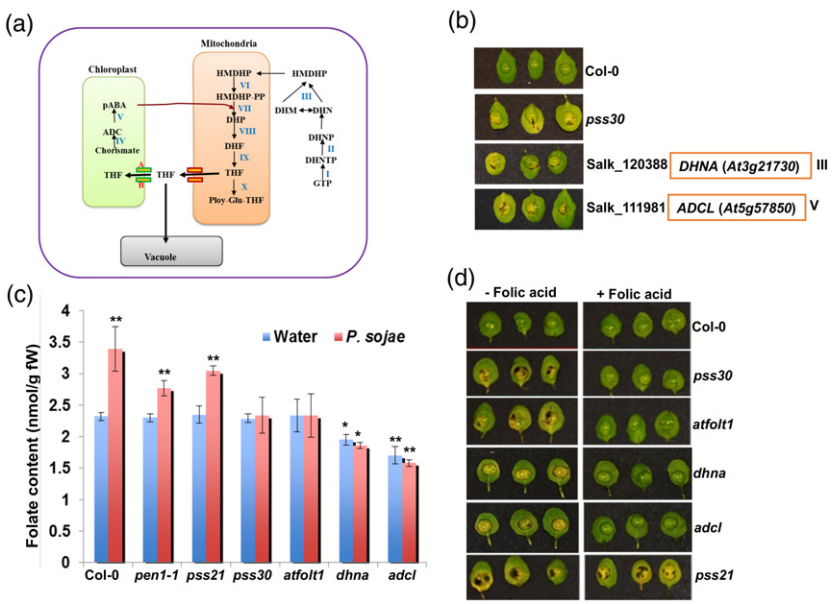

Figure 2. Folate is involved in non-host immunity.
(a) Folate biosynthesis pathway in plants (Hanson and Gregory, 2011). Roman numbers denote enzymes of the individual steps of the folate biosynthesis pathway: I, GTP (guanosine-5′-triphosphate) cyclohydrolase 1; II, dihydroneopterin (DHN) triphosphate diphosphatase; III, DHN aldolase (DHNA); IV, 4-amino-4-deoxychorismate synthase; V, 4-amino-4-deoxychorismatelyase (ADCL); VI, 6-hydroxymethyldihydropterin (HMDHP) pyrophosphokinase; VII, dihydropteroate (DHP) synthase; VIII, dihydrofolate (DHF) synthase; IX, DHF reductase; X, isoforms of folylpolyglutamate synthase. ADC, aminodeoxychorismate; DHM, dihydromonapterin; Glu, glutamate; P, phosphate; pABA, *p*-aminobenzoate; THF, tetrahydrofolate. A in red font indicates AtFOLT1; B in red font indicates AtFOLT2, the folate transporter encoded by *At2g32040*. (b) Loss of Arabidopsis non-host resistance in the two T-DNA insertion folate biosynthesis knockout mutants against *P. sojae*: (i) mutant for the *At3g21730* gene, encoding DHN aldolase (DHNA) (III); and (ii) mutant for the *At5g57850* gene, encoding 4-amino-4-deoxychorismate lyase (ADCL) (V). (c) Complementation of non-host immunity against *P. sojae* among the folate transporter and folate biosynthesis mutants following external feeding of roots with 5 mM folic acid. (d) Induced accumulation of folate in Arabidopsis following *P. sojae* infection. Values show the mean ± SE of six individual experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to water-treated Col-0. fw, fresh weight of leaves.

mapped to the *PSS1* region were considered candidate *PSS30* genes (Figure 1c; Table 1). We evaluated transfer DNA (T-DNA) insertion mutants generated in the Col-0 ecotype for each of the three candidate *PSS30* genes for responses to *P. sojae* (Table S2). Two independent mutants carrying a T-DNA insertion in the *At5g66380* gene were shown to be susceptible to *P. sojae* (Figure 1e). Therefore, *At5g66380* is most likely the *PSS30* gene (Figure 1c–e). Complementation of the T-DNA insertion mutants with a CaMV 35S promoter-fused *At5g66380* gene confirmed that *At5g66380* is the *PSS30* gene (Figure 1e). *At5g66380* encodes a folate transporter, which was previously localized to the chloroplast envelope and named as AtFOLT1 (Bedhomme et al., 2005). Reverse transcriptase-PCR (RT-PCR) demonstrated that the complemented mutant plants but not the two T-DNA insertion knockout mutants contained *AtFOLT1* transcripts (Figure 1f).

To determine if the EMS-induced mutation in *At5g66380* was responsible for loss of immunity in the *pss30* mutant, we generated F_1 plants by crossing *pss30* with *atfolt1-1* (Figure 1g,h; Figure S3). To determine the responses of the F_1s, *pss30* and *atfolt1-1* plants to *P. sojae*, we conducted quantitative PCR (qPCR) for the *P. sojae* actin gene *ActA* to assess the relative biomass levels of the pathogen among the genotypes following infection with *P. sojae*. Significantly increased *P. sojae* biomass levels were detected in the two mutants as well as in F_1s as compared to that in Col-0 or the complemented *atfolt1-1* mutant (Figure 1h; Figure S3). The F_1 plants failed to show any complementation between the two mutants and were susceptible to *P. sojae*. These

results confirmed that mutation in *At5g66380* is responsible for the loss of immunity observed in the *pss30* mutant. The EMS-induced *pss1* mutant is more susceptible to *P. sojae* than the T-DNA insertion *atfolt1-1* mutant (Figure 1h). The T-DNA insertion *atfolt1-1* mutant and the complemented *atfolt1-1* mutant through overexpression of *AtFOLT1/PSS30* showed normal growth and development with no obvious morphological changes.

Folate contributes to non-host immunity

Based on the observation that a lack of the *AtFOLT1* folate transporter gene resulted in the loss of Arabidopsis non-host immunity against two soybean pathogens, we hypothesized that folate may contribute to plant immunity. Plants synthesize folate in three organelles: (i) pterin is synthesized in the cytosol, (ii) *p*-aminobenzoate (pABA) is synthesized in plastids and (iii) pterin and pABA are coupled and glutamylated to folate in mitochondria (Hanson and Gregory, 2011). Folate is subsequently transported into the cytosol, plastids and vacuoles (Hanson and Gregory, 2011; Figure 2a). To test our hypothesis, Arabidopsis T-DNA insertion folate biosynthesis mutants were investigated for possible loss of non-host immunity (Table S3). Study of insertion mutants in genes involved in folate biosynthesis (Figure 2a) revealed a loss of non-host immunity against *P. sojae* among T-DNA insertion mutants of two folate biosynthesis genes that encode the key regulatory enzymes dihydroneopterin aldolase (DHNA) and 4-amino-4-deoxychorismatelyase (ADCL), localized to the cytoplasm and chloroplasts, respectively (Figure 2b; Figures S4 and S5).

We observed that following *P. sojae* infection, steady-state folate levels were increased in the wild type (Col-0) and in *pen1-1* and *pss21* mutants but not in the two folate transporter mutants (Figure 2c). The lack of induced folate accumulation in the *atfolt1* or *pss1* mutants following *P. sojae* infection suggests that transport of folate presumably to the chloroplast is impaired in these two mutants. Two folate biosynthesis mutants, *dhna* and *adcl*, lacking non-host immunity to *P. sojae* also exhibited reduced folate levels (Figure 2c).

We determined if exogenous feeding of *pss30*, *atfolt1*, *dhna* and *adcl* mutants with folic acid (a synthetic analog of folate) at high concentrations could prevent the loss of non-host immunity in the four mutants. Each of the mutants expressed immunity against *P. sojae* following external feeding with folic acid (Figure 2d). The lack of induced folate accumulation in *atfolt1* and *pss30* mutants following infection due to the absence of the AtFOLT1 transporter and complementation of the folate transporter mutants with exogenous feeding of folic acid at high concentrations indicate that passive transport of folic acid into the plastids may have contributed to the recovery of the lost non-host immunity function among the folate transporter mutants (Figure 2c,d). This complementation of the impaired non-host immunity was specific to the folate transporter and folate biosynthesis mutants (Figure 2d). No rescue of the impaired non-host immunity function of the Arabidopsis *pss21* mutant was observed upon external feeding with folic acid because in this mutant the AtFOLT1 transporter is functional and there is no defect in the folate biosynthesis pathway.

Overexpression of *AtFOLT1* enhances resistance of transgenic soybean lines against *F. virguliforme*

We have demonstrated that the Arabidopsis *pss30* mutant is susceptible to the fungal pathogen *F. virguliforme*, which causes foliar sudden death syndrome (SDS) and root rot in soybean. We therefore investigated if overexpression of *AtFOLT1* could enhance disease resistance in soybean by increasing the folate content during pathogen infection. Three fusion genes (Figure S6) were generated to express *AtFOLT1* in soybean by fusing *AtFOLT1* to (i) the *CaMV* 35S promoter, (ii) the promoter (Prom 1) of the infection-inducible soybean *Glyma18g47390* gene encoding an *S*-adenosyl-l methionine:carboxyl methyltransferase (Ngaki et al., 2016, 2020; Sahu et al., 2017) and (iii) the root-specific promoter (Prom 2) from the soybean *Glyma10g31210* gene (Gunadi et al., 2016; Lanubile et al., 2015; Ngaki et al., 2020). Multiple independent transgenic soybean lines were developed for each of the three fusion genes and evaluated for possible enhanced SDS resistance against *F. virguliforme*.

We observed enhanced foliar SDS resistance among the transgenic soybean lines as compared to the non-transgenic transgene-recipient soybean line 'Williams 82' under growth chamber conditions (Figure 3a). Enhanced SDS resistance was associated with the expression of the transgenes and increased folate steady-state levels in infected roots (Figure 3b,c). Although an increase in folate concentration was induced in the roots of non-transgenic Williams 82 plants following *F. virguliforme* infection, significantly higher folate levels were recorded for the transgenic lines as compared to the non-transgenic control following *F. virguliforme* infection (Ti/Wi comparison in Figure 3c). Three selfed generations of the transgenic lines were evaluated in 2015, 2016 and 2018 under field condition for possible enhanced SDS resistance. Significantly enhanced SDS resistance as compared to the non-transgenic Williams 82 line was observed in all 3 years among some lines carrying *AtFOLT1* fused to CaMV 35S and Prom2 promoters (Figure 3d,e).

Overexpression of *AtFOLT1* enhances soybean cyst nematode resistance

It was reported earlier that the dominant soybean cyst nematode (SCN) resistance gene *Rhg4* encodes a serine hydroxyl methyl transferase (SHMT) that metabolizes

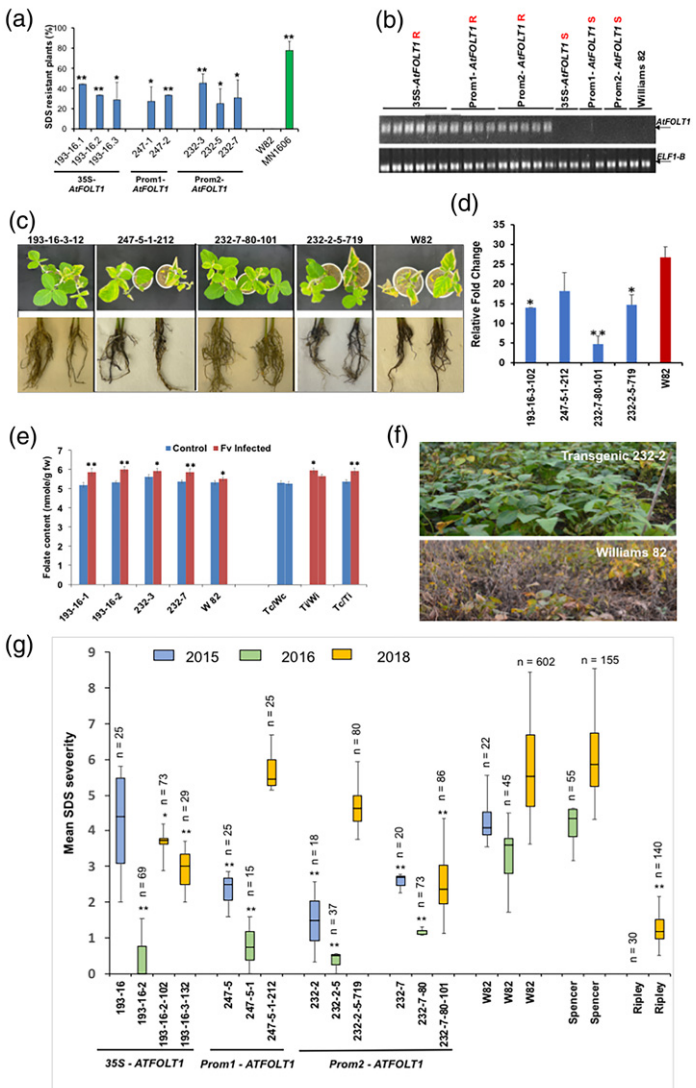

Figure 3. Overexpression of *AtFOLT1* enhances resistance of transgenic soybean lines against *F. virguliforme*.
(a) Independent transgenic soybean lines carrying *AtFOLT1* showed enhanced resistance to *F. virguliforme* under growth chamber conditions. Foliar sudden death syndrome (SDS) symptoms were measured using a quantitative scale ranging from 1 to 7, with 1 indicating no symptoms and 7 indicating death of plants (see the Experimental procedures section for details). Plants with foliar SDS scores of 2 or less were considered resistant, and those with scores of >2 were considered susceptible. Data are shown as the mean ± SE of disease scores from 10 replications. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the non-transgenic recipient Williams 82 (W 82). MN1606 is an SDS-resistant soybean line. (b) Expression of *AtFOLT1* in roots of *F. virguliforme*-infected transgenic soybean plants detected by RT-PCR. Each genotype is identified with either R or S in red font to show their responses to *F. virguliforme*. R, resistant to *F. virguliforme*; S, susceptible to *F. virguliforme*. *ELF1B* was used as an internal control. (c) Foliar and root SDS phenotypes at 3 weeks post-infection. (d) The relative fungal biomass was calculated as the amplification of the fungal *FvTox1* gene relative to that of the soybean gene *Glyma.05G014200* (internal control) by qPCR. Data are shown as the average ± SE of three independent experiments ($n = 18$ plants per line per experiment). $^{*}P < 0.05$ and ** at $P < 0.01$ compared with Williams 82. (e) Folate contents in the roots of transgenic and control lines. Blue bars, transgenic lines with no *F. virguliforme* infection; red bars, following *F. virguliforme* infection. Data are shown as the mean ± SE of six replications. Tc, mean of averages of all transgenic lines with no infection; Ti, mean of averages of all transgenic lines following *F. virguliforme* infection; Wc, Williams 82 with no infection; Wi, Williams 82 following *F. virguliforme* infection. $^{*}P < 0.05$ and $^{**}P < 0.01$ due to *F. virguliforme* infection within each individual soybean line. Tc/Wc, comparison of mean data of all transgenics (Tc) with Williams 82 (Wc) without *F. virguliforme* infection. Ti/Wi, comparison of the mean of averages of all transgenics (Ti) with that of Williams 82 (Wi) following *F. virguliforme* infection. Tc/Ti, comparison of the mean of averages of all transgenic lines (Tc versus Ti) without or with *F. virguliforme* infection. fw, fresh weight of roots. $^{*}P < 0.05$ and $^{**}P < 0.01$. (f) Enhanced foliar SDS resistance in a transgenic soybean line and susceptibility in the transgene-recipient soybean line Williams 82 under field conditions in 2015. (g) Enhanced SDS disease resistance among transgenic soybean lines carrying *AtFOLT1* transgenes in field trials conducted in 2015 (blue bars), 2016 (red bars) and 2018 (green bars). Transgenic lines 193, 247 and 232 carry *PSS30/AtFOLT1* in the recipient cultivar Williams 82 (W82). 'Ripley' and 'Spencer' are SDS-resistant and -susceptible soybean cultivars, respectively, used as control lines during 2016 and 2018. In 2015, segregating R_1 progenies, and in 2016 and 2018, homozygous R_2 and R_3 plants, respectively, were evaluated. Plants were scored with a scale of 0–9, with 0 indicating no SDS and 9 indicating most SDS. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the non-transgenic control Williams 82 in the same year; *n* is the total number of plants evaluated for foliar SDS. Data are shown as mean ± SE of three replications.

folate to 5,10-methylenetetrahydrofolate. An altered structure of SHMT encoded by the *rhg4* allele causes SCN susceptibility (Liu et al., 2012). The *AtFOLT1* transgene-recipient Williams 82 line carries the *rhg4* allele and is SCN-susceptible. Since overexpression of *AtFOLT1* significantly enhances folate levels following *F. virguliforme* infection among the transgenic lines (Ti/Wi comparison in Figure 3(d), we hypothesized that the folate levels would be elevated in the roots of *AtFOLT1*-transgenic soybean plants following SCN infection. The increased folate levels among the transgenic soybean plants following SCN infection might then generate SCN resistance by compensating for the reduced activity of the SHMT enzyme encoded by the *rhg4* allele. We therefore investigated if the transgenic soybean lines carrying the *AtFOLT1* transgenes exhibit enhanced SCN resistance as compared to the transgene-recipient Williams 82 line. Enhanced SCN resistance was observed among the transgenic lines overexpressing *AtFOLT1* (Figure 4a–c). Increased folate levels with enhanced SCN resistance were observed among the transgenic soybean lines (Figure 4d). Among the transgenic soybean lines overexpressing *AtFOLT1*, folate levels were increased approximately 12% following SCN infection (Figure 4d) as compared to the SCN-infected non-transgenic Williams 82 control plants. Presumably, a 12% increase in the average folate levels among the transgenic lines was sufficient to compensate the reduced activity of the SHMT isoform encoded by the *rhg4* allele in Williams 82 and to enhance SCN resistance among the transgenic soybean lines (Figure 4c).

Overexpression of *AtFOLT1* results in early onset of senescence among the transgenic soybean lines

In this study, we used three promoters for expression of the Arabidopsis *AtFOLT1* gene in soybean. No obvious altered phenotypes were observed for transgenic soybean lines carrying any of the three fusion *AtFOLT1* genes except the one generated by fusing *AtFOLT1* with the CaMV 35S promoter. We observed early senescence among the transgenic lines carrying the CaMV 35S promoter-fused *AtFOLT1* gene under field conditions. As a result, the plants did not attain the height observed for the transgenic lines carrying the other two fusion *AtFOLT1* genes or the recipient Williams 82 line. Despite reduced plant height, we did not see any negative impact of early senescence on yield per plant (Figures S7 and S8).

DISCUSSION

In this study we have shown that mutations in the Arabidopsis *PSS30* gene cause loss of non-host immunity against two non-adaptive soybean pathogens, *P. sojae* and *F. virguliforme*. Through map-based cloning, we have demonstrated that *PSS30* encodes the AtFOLT1 folate transporter, localized earlier to the chloroplast envelope (Figure 1; Bedhomme et al., 2005). The non-synonymous mutation detected in the *At5g66380* gene of the *pss30* mutant resulted in substitution of a leucine residue with an isoleucine residue at position 66 and susceptibility to *P. sojae* and *F. virguliforme* (Figure 1d; Table 1; Figure S3). Similar isoleucine-to-leucine substitutions have been shown to result in changes in gene structure and function (Christoffers et al., 2002; Délye et al., 2002; Sitbon et al., 1991; Vipond et al., 1996; Wu et al., 2015).

In Arabidopsis, in addition to *AtFOLT1*, another gene, *At2g32040*, also encodes a folate transporter localized to the chloroplast (Hanson and Gregory, 2011). It was reported that in the absence of a functional AtFOLT1, the chloroplast folate levels are altered although plant growth and development remain normal (Bedhomme et al., 2005). In our study the *atfolt1* mutants and complemented mutants through overexpression of *AtFOLT1/PSS30* also showed normal growth and development with no obvious morphological changes.

We hypothesized that the absence of AtFOLT1 folate transport into chloroplasts results in loss of non-host immunity. To test this hypothesis we investigated mutants of folate biosynthesis pathway genes. Folate biosynthesis *adcl* and *dhna* mutants with reduced folate levels showed a loss of non-host immunity against *P. sojae*, thus confirming that folate contributes to non-host immunity (Figure 2b). However, the reduction in folate levels in these two mutants was not striking. Several enzymes of the folate biosynthesis pathway are encoded by more than one gene (Table S3). Of the two mutants showing loss of non-host immunity, two putative homologues were identified for the gene *DHNA*, encoding DHNA (Table S3). This gene redundancy may be responsible for the small reduction in steady-state folate levels in the *dhna* mutant. In the *adcl* mutant, although we observed much reduced folate accumulation as compared to the *dhna* mutant (Figure 2), considering a possible lack of its homologue (Table S3; Gorelova et al., 2019), there may be an unknown mechanism for compensating its loss of ADCL function. Folate biosynthesis is highly complex and the biochemistry of folate biosynthesis in plants is not completely understood (Gorelova et al., 2019; Hanson and Gregory, 2011).

Folate rapidly accumulates following *P. sojae* infection in both host and non-host plants (Figure 2; Figure S9). Exogenous application of folic acid can reverse the loss of non-host immunity function of the *pss30*, *atfolt1* and folate biosynthesis mutants (Figure 2c). Complementation of *pss30* and *atfolt1* through passive transport of exogenously applied folic acid also suggests that active transport of folate through the AtFOLT1 transporter into chloroplasts is essential for non-host immunity in Arabidopsis.

To further investigate the contribution of folate in disease resistance, AtFOLT1 was overexpressed in soybean. Enhanced resistance of soybean plants to both

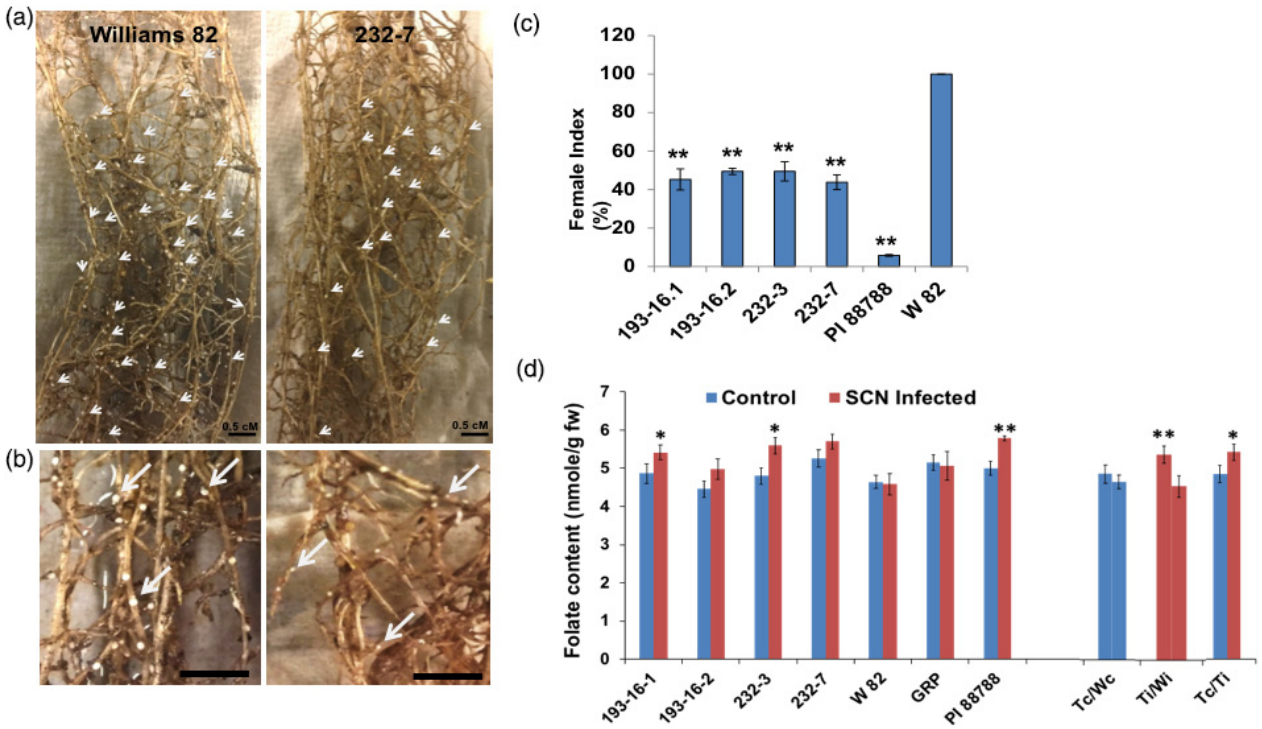

Figure 4. Overexpression of *AtFOLT1* enhances resistance against SCN among transgenic soybean lines.
(a) Enhanced SCN resistance of a transgenic soybean line. The SCN cysts on soybean roots are shown with arrows. Lower numbers and smaller cysts were observed in the roots of the 232-7 transgenic line (on the right) compared to the susceptible non-transgenic control, Williams 82 (on the left). (b) Close-up of the cysts formed on roots shown in (a). (c) Responses of soybean roots to SCN HG type 2.5.7. Female indices (FIs) among the transgenic soybean lines were calculated considering Williams 82 as the susceptible line. Data are shown as the mean ± SE of 10 replications. $^{**}P < 0.01$ compared to Williams 82 (W 82). (d) Folate levels of the soybean roots. Data are shown as the mean ± SE of six replications. Blue bars, soybean roots without SCN infection; red bars, following SCN infection. $^{*}P < 0.05$ and $^{**}P < 0.01$. Tc/Wc, comparison of mean data of all transgenics (Tc) with Williams 82 (Wc) without SCN infection. Ti/Wi, comparison of the mean of averages of all transgenics (Ti) with that of Williams 82 (Wi) following SCN infection. Tc/Ti, comparison of the mean of averages of all transgenic lines (Tc versus Ti) without or with SCN infection. $^{*}P < 0.05$ and $^{**}P < 0.01$. GRP, a transgenic soybean line that carries an Arabidopsis *glycine-rich protein 1* gene (Wang et al., 2018). fw, fresh weight of roots.

F. virguliforme and *H. glycines* was observed among most of the transgenic soybean plants expressing the Arabidopsis *AtFOLT1* gene (Figures 3 and 4). The transgenic soybean lines with enhanced broad-spectrum resistance showed increased steady-state folate levels (Figures 3 and 4). Increased folate levels among the transgenic soybean lines are suspected to be involved in moderating the effects of the SHMT defect in folate metabolism in the transgene-recipient cultivar Williams 82 and thereby conferring broad-spectrum resistance through an unknown mechanism. Mutation in the Arabidopsis *SHMT1* gene, encoding serine hydroxymethyl transferase involved in the photorespiratory pathway, results in chlorotic and necrotic lesion formation under a variety of environmental conditions and loss of immunity against both biotrophic and necrotrophic pathogens (Moreno et al., 2005).

Induction of host immunity following external application of folic acid, a synthetic analog of folate, is well documented (Boubakri et al., 2016; Lenk et al., 2018; Wittek et al., 2015). A transcriptomic study of transgenic rice (*Oryza sativa*) lines engineered for increased folate content revealed that 11 of the 111 induced genes encode putative disease resistance NBS-LRR-type receptor proteins and three encode putative lipid transfer proteins, which could be involved in plant defenses (Blancquaert et al., 2013). Here we have shown through a study of mutants that folate contributes to non-host immunity. We have also shown that overexpression of a folate transporter enhances folate accumulation following pathogen infection and generates broad-spectrum disease resistance (Figures 3 and 4).

Cytological and immunoblotting studies showed that AtFOLT1 is localized to the chloroplast envelope (Bedhomme et al., 2005). It was surprising to observe that in spite of *atfolt1* and *pss30* mutants lacking the functional *AtFOLT1* transporter gene but containing all of the genes involved in the folate metabolic biosynthesis pathway, they failed to enhance accumulation of folate following *P. sojae* infection as in the wild-type Col-0 ecotype and the *pss21* mutant (Figure 2c). In contrast, overexpression of AtFOLT1

enhanced folate accumulation in roots of transgenic soybean plants as compared to the transgene-recipient Williams 82 plants following *F. virguliforme* and *H. glycines* infection (Figures 3d and 4d). These observations suggest that the AtFOLT1 transporter plays a critical role in folate accumulation following *P. sojae* infection. Lack of the AtFOLT1 folate transporter leads to suppression of the infection-specific folate accumulation in Arabidopsis *pss30* and *atfolt1-1* mutants (Figure 2d) and overexpression of *AtFOLT1* in soybean results in enhanced infection-specific folate accumulation among the transgenic soybean lines (Figures 3d and 4d).

We hypothesize that in the absence of the AtFOLT1 transporter, active transport of folate from the cytoplasm into chloroplasts is compromised and therefore folate levels in the cytoplasm remain unaltered in the *pss30* and *atfolt1-1* mutants following *P. sojae* infection. In the wild-type Col-0 ecotype or *pss21* mutant with a functional AtFOLT1 transporter, folate is actively transported into chloroplasts following infection and as a result, the cytoplasmic folate level is reduced. Folate may have a feedback inhibition on its own biosynthesizing enzyme(s) and depletion of folate levels in the cytoplasm is essential for relieving this feedback regulation for inducing folate biosynthesis and accumulation following *P. sojae* infection. During pathogen infection, folate is actively transported into the chloroplast, relieving the negative feedback regulation of folate on its own biosynthesis in the cytoplasm, which results in enhanced folate accumulation (Figure 2d; Figure S6).

Feedback repression and feedback activation mechanisms at different steps of the folate biosynthesis pathway have been reviewed previously (Hanson and Gregory, 2011). An *in vitro* study has revealed that dihydropteroate synthase (DHPS), an intermediate enzyme of the folate biosynthesis pathway (Figure 2a), is competitively inhibited by the products dihydropteroate, dihydrofolate (DHF) and tetrahydrofolate (THF) (Hanson and Gregory, 2011; Prabhu et al., 1997). It has been shown that one of the three dihydrofolate reductase-thymidylate synthase isoforms inhibits the function of the other two dihydrofolate reductase-thymidylate synthase isoforms in order to regulate THF accumulation (Gorelova et al., 2017). Based on the observations in this study and previous reports (Gorelova et al., 2017; Hanson and Gregory, 2011), we propose that folate exerts an *in vivo* negative feedback regulation on its own biosynthesis. This negative feedback regulation is however relieved following its transport from the cytoplasm to chloroplasts during pathogen attacks. This results in folate biosynthesis and accumulation, leading to non-host immunity. It appears that folate accumulation is tightly regulated, and negative feedback regulation could control its biosynthesis and accumulation during interaction of a non-host plant species with a non-adapted pathogen.

In this study, we observed early senescence among the transgenic lines carrying the CaMV 35S promoter fused to the *AtFOLT1* gene under field conditions. As a result, the plants did not attain the height observed for the transgenic lines carrying the other two fusion *AtFOLT1* genes or the transgene-recipient Williams 82 line. In a recent study in rice it was shown that a mutation in the *HPA1* gene, encoding a tetrahydrofolate deformylase, resulted in increased H_2O_2 accumulation and early leaf senescence (Xiong et al., 2020). They found that the functions of mitochondrial oxidative phosphorylation complex I and complex V were affected by the *hpa1* mutation, resulting in enhanced H_2O_2 accumulation and leaf senescence. HPA1's absence led to accumulation of tetrahydrofolate, 5-formyl tetrahydrofolate and 10-formyl tetrahydrofolate and a reduction in the expression of tetrahydrofolate cycle-associated genes encoding SHMT, glycine decarboxylase and 5-formyl tetrahydrofolate cycloligase (Xiong et al., 2020).

Folate-dependent tRNA methylation is essential for mitochondrial translation. SHMT2 synthesizes 5,10-methylenetetrahydrofolate, which is a methyl donor for taurinomethyluridine used at the wobble position of selected mitochondrial tRNAs. When SHMT2 was knocked out in a human cell line, mitochondrial translation was stalled for respiratory chain enzymes, leading to impaired oxidative phosphorylation in human cells (Morscher et al., 2018).

Senescence among the 35S-*AtFOLT1* plants occurred over 2 weeks earlier than in the wild-type Williams 82 lines. As the plant ages, the folate levels decline (Bedhomme et al., 2005). We hypothesize that induced early senescence among the *AtFOLT1*-overexpressing transgenic soybean plants is caused by reduced folate levels in mitochondria resulting from increased transport of folate from the cytoplasm into chloroplasts in the 35S-*AtFOLT1* plants as compared to that in the non-transgenic Williams 82, Prom1-PSS30 or Prom2-PSS30 lines (Figure S7). Among the aging transgenic soybean plants, enhanced active folate transport into chloroplasts due to overexpression of the AtFOLT1 transporter by the CaMV 35S promoter might have caused significant reduction in folate levels in mitochondria. Shuttle reduction in folate levels in mitochondria among the aging soybean leaves may have changed the oxidative phosphorylation status, leading to early senescence (Bedhomme et al., 2005; Xiong et al., 2020).

Folate acts as a cofactor for SMHT in the interconversion of glycine to serine. In one-carbon (1C) metabolism, folate is also a cofactor for thymidylate synthase (TYMS), which converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by using 5,10-methylene-THF. The synthesis of dTMP is important for DNA replication and repair (Ducker and Rabinowitz, 2017; Ozer et al., 2015). The inhibition of TYMS induces

accumulation of intracellular ROS, leading to oxidative stress (Ducker and Rabinowitz, 2017; Ozer et al., 2015).

It has also been shown that folate metabolism contributes to production of NADPH through catalysis of 5,10-methylene-THF to 5,10-methenyl-THF by methylenetetrahydrofolate dehydrogenase (MTHFD) to maintain the redox balance to protect plants against oxidative stress (Gorelova et al., 2017). Production of ROS is crucial for the hypersensitive response (HR) in plants. However, excessive ROS production leads to failure in containment of the HR, providing avenues for pathogens to cause diseases (Moreno et al., 2005). SHMT is one of the key photorespiratory enzymes, and is essential for preventing excess ROS production in the chloroplast to protect plant cells from oxidative damage and limiting the HR in order to avoid any pathogen establishment and disease development (Moreno et al., 2005; Wingler et al., 2000).

In roots, the plastids are colorless and known as leucoplasts. They do not have any photosynthetic apparatus, primarily store foods and have an active folate biosynthesis pathway. A mutant study has revealed that a plastidial isoform of folylpolyglutamate synthetase (FPGS) that catalyzes the addition of glutamate residues to the folate molecule to form folylpolyglutamates is essential for normal development of the quiescent center, the auxin gradient in the root cap and the actin cytoskeleton for cell division and expansion during post-embryonic root development in Arabidopsis (Srivastava et al., 2011). *AtFOLT1*, encoding the plastidial transporter, is reported to be constitutively expressed including in roots (Bedhomme et al., 2005). In this investigation we have shown that overexpression of AtFOLT1 in soybean roots causes enhanced accumulation of folate following pathogen infection (Figures 3e and 4d).

In soybean, the SCN resistance gene *Rhg4* encodes an SHMT, GmSHMT08 (Liu et al., 2012). Recently, it has been shown that non-sense mutations leading to truncated GmSHMT08 proteins failed to confer SCN resistance in two distinct mutants (Kandoth et al., 2017). The *rhg4* allele in the transgene-recipient Williams 82 is identical to the *rhg4* allele identified earlier from the SCN susceptible cultivar 'Essex' and is distinct from the *Rhg4* allele of the SCN-resistant cultivar 'Forrest' (Liu et al., 2012). Investigation of the *in vitro* activities of the Essex and Forrest SHMT08 isoforms encoded by the alleles at the *Rhg4* locus revealed typical Michaelis–Menten kinetics for the Forrest SHMT08 isoform but not for the Essex SHMT08 isoform (Liu et al., 2012). Increased folate levels following SCN infection among the *AtFOLT1*-overexpressing Williams 82 transgenic lines most likely improved the function of Essex SHMT08 isoform encoded by the *rhg4* allele to confer enhanced SCN resistance. It is unclear if the enhanced SDS resistance among the *AtFOLT1*-overexpressing Williams 82 transgenic lines is induced by the same genetic mechanism as proposed for enhanced SCN resistance. Further study is warranted to determine if the overexpressed *AtFOLT1* gene can enhance SDS resistance in the *Rhg4* genetic background.

Contrary to evidence in support of a positive role for folate and the folate-metabolizing enzyme SHMT in host and non-host resistance against biotrophic, hemibiotrophic and necrotrophic pathogens (Liu et al., 2012; Moreno et al., 2005; Figures 1–4), involvement of folate in enhancing susceptibility of Arabidopsis against the bacterial pathogen *Pseudomonas syringae* pv. glycinea DC3000 (*Pst*) through epigenetic regulation has been reported (Gonzalez and Vera, 2019). It has been demonstrated that a methionine synthase generates methionine through folate-dependent 1C metabolism and contributes to production of *S*-adenosyl methionine for genome-wide methylation. Overexpression of the methionine synthase led to enhanced genome-wide methylation and repressed resistance of Arabidopsis against *Pst* (Gonzalez and Vera, 2019). These conflicting observations among different plant–pathogen interactions suggests that multiple mechanisms are regulated by folate and folate-mediated 1C metabolism for expression of plant immunity.

Folate is essential for human health and its deficiency can cause severe health consequences, especially during fetal development. A contribution of folate to maintenance of the regulatory T (Treg) cells involved in suppression of the autoimmunity response has been documented (Bettelli et al., 2006). In the absence of folate, native T cells can differentiate into Treg cells. However, folate is essential for maintenance of Treg cells. Decreased expression of anti-apoptotic molecules (Bcl-2) in the absence of folate is responsible for Treg cell death (Kunisawa et al., 2012). It is however unknown if folate contributes to innate and adaptive immunity in humans. It will be interesting to investigate if there is any conservation of the role of folate in immunity across the kingdoms.

EXPERIMENTAL PROCEDURES

Plant materials and growing conditions

Arabidopsis T-DNA mutants and Columbia (Col-0) and Niederzenz (Nd-0) ecotypes used in this work were obtained from the Arabidopsis Biological Resources Center (ABRC; Columbus, OH, USA). *pen1-1* seeds were kindly provided by Dr. Paul Schulze-Lefert. Arabidopsis seeds were sown on LC1 soilless mixture (Sun Gro Horticulture, Bellevue, WA, USA). Seeds were incubated at 4°C for 2 days to break the dormancy prior to planting. Ten-day-old seedlings were transplanted and grown at 22 ± 1°C under 60–80 μmol photons m^{-2} sec^{-1}, at 60% relative humidity and under a 16 h light/8 h dark photoperiod. Soybean lines Williams, Williams 82, PI 88788 and MN1606 were used for this study. Transgenic and non-transgenic soybean plants were grown in greenhouse and growth chambers for seed collection and bioassays, respectively. Williams and Williams 82 cultivars were grown in the dark for 7 days under conditions described previously (Ward et al., 1979).

Pathogens and inoculation

Detached leaves of 3-week-old Arabidopsis plants were used for inoculation with *P. sojae* zoospores (3×10^5/ml) as described earlier (Sumit et al., 2012). Detached leaves of 3-week-old Arabidopsis plants were inoculated with *F. virguliforme* conidiospores (10^6 spores/ml) incubated for 48 h under conditions described earlier for *P. sojae* inoculation (Sumit et al., 2012). Over 24 plants from each F_3 family were inoculated with *P. sojae* for mapping the *PSS30* gene. The homozygous resistant and susceptible F_3 families and those segregating were identified based on the proportion of resistant and susceptible plants in individual F_3 families. Families with less than 15% and more than 70% susceptible progenies were scored as homozygous resistant and susceptible families, respectively. Families with 16–69% susceptible progenies were scored tentatively as heterozygous. Bulked segregant analysis (BSA) was conducted using 10 susceptible families (Michelmore et al., 1991; Figure S2a). Based on BSA and initial mapping using homozygous resistant and susceptible families, *PSS30* linked markers were identified. One of those markers, SBP5_25.5, was used to characterize the homozygous and heterozygous families (Figure 1). Five families carrying only susceptible progenies and showing the *pss30*-specific SBP5_25.5 allele were selected for resequencing (described below).

Hypocotyls of 7-day-old etiolated soybean seedlings were inoculated with zoospores of the *P. sojae* CC-5C isolate (Ducker and Rabinowitz, 2017). Tissues were harvested at different time periods, immediately frozen in liquid nitrogen and stored at –80°C for RNA extraction and folate measurement.

For preparation of *F. virguliforme* inoculum, 500 g of overnight soaked sorghum (*Sorghum bicolor*) grains in two-quart Mason jars was autoclaved for 40 min at 121°C. Sterilized sorghum grains in jars were inoculated with *F. virguliforme* spores by transferring ten 20-mm-diameter plugs from 2-week-old culture maintained on 1/3 PDA plates. The jars were then incubated under sterile conditions under room light and temperature conditions and shaken every other day for 2 weeks to ensure uniform fungal growth. After 1 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 (Luckew et al., 2012).

SCN HG type 2.5.7-infested soil collected from Muscatine, IA, USA was used to fill cone-tainers for planting soybean seeds. Soil in each cone-tainer contained approximately 50 SCN cysts for determining responses of soybean lines to the pathogen. The HG type was classified according to a published report at the SCN Diagnostics Center (University of Missouri-Columbia) (Niblack et al., 2002).

Flg22 treatment

For flg22 treatment, leaves of 5-week-old Arabidopsis were syringe-infiltrated with either 1 μM flg22 in water or water alone. After 24 h, tissue samples were harvested and frozen in liquid nitrogen to extract the RNA and determine the folate content.

Mapping *PSS30*

Genomic DNA was isolated from Arabidopsis using a CTAB method (Weigel and Glazebrook, 2002). An equal amount of DNA (approximately 10 μg) was mixed from 10 individual $F_{2:3}$ families for BSA (Michelmore et al., 1991). PCR was performed using SSLP, dCAPS and sequence-based polymorphic (SBP) markers covering the Arabidopsis genome. PCR conditions for SSLP, dCAPS and SBP markers were similar to the ones described earlier (Sahu et al., 2012; Sumit et al., 2012). Primers for molecular markers are listed in Table S4.

Microscopy

Detached leaves of 3-week-old Arabidopsis plants were infected with *P. sojae* and stained with Trypan blue dye at 3 or 5 days post-inoculation and destained in chloral hydrate (Koch and Slusarenko, 1990). Samples were then examined under a Zeiss Axioplan II compound microscope (Zeiss Inc., Thornwood, NY, USA) equipped with a Cannon G10 digital camera.

Identification of candidate *PSS30* genes

Genomic DNA was isolated from five homozygous susceptible *pss30pss30* $F_{2:3}$ families using a CTAB method and mixed in equal amounts (Weigel and Glazebrook, 2002). The bulked DNA of five *pss30pss30* $F_{2:3}$ families was used to conduct whole-genome sequencing using an Illumina HiSeq 3000 platform at the DNA Facility, Iowa State University. After filtering, the 100-bp paired-end sequence reads were aligned to the reference Col-0 sequence using the SHORE program for identification of candidate mutations in the *PSS30* region (Ossowski et al., 2008; Schneeberger et al., 2009). Mutations common to *pss30* and *pen1-1* in the *PSS30* region were not studied further. Non-synonymous or non-sense mutations of the *PSS30* region were considered to identify candidate *PSS30* genes.

RT-PCR, qRT-PCR and qPCR analyses

Total RNA samples were extracted from Arabidopsis leaf and soybean root tissues using the miRNeasy Mini Kit (Qiagen, Germantown, MD, USA). RNAs were treated with DNase I (Invitrogen, Inc., Carlsbad, CA, USA) to remove genomic DNA. RNAs were quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and their quality was evaluated by separation on a 0.8% agarose gel. cDNAs for RT-PCR were synthesized from 2 μg of total RNAs using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's recommendations. RT-PCR was conducted for selected genes (Table S4) using cDNAs prepared from infected or water-treated tissues of either Arabidopsis or soybean. The PCR program was as follows: initial denaturation at 94°C for 3 min, followed by 32 cycles of 94°C for 30 sec, 52 or 55°C for 1 min and 72°C for 1 min and one final extension step at 72°C for 10 min. *ACTIN* or *GmELF1B* was used as an internal control for Arabidopsis and soybean, respectively.

qPCR was performed for Arabidopsis genes using SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) in an iCycler Real-Time system (Bio-Rad) for 32 cycles of 95°C for 30 sec, 58°C for 1 min and 72°C for 1 min. Each reaction was performed thrice, and expression levels for all genes were normalized using *ACTIN* as an internal standard. All samples were subtracted against a calibrator sample, the water-treated wild type (Col-0). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Kambakam et al., 2016; Sekhar et al., 2010). The *P. sojae* biomass in F_1 hybrid plants was quantified using genomic DNA qPCR as follows. DNA was diluted to 20 ng μl^{-1} and primers specific to the *P. sojae actin* gene were used to PCR amplify and measure the fungal biomass. The Arabidopsis *AtUbi-5* gene was used as the internal control (Minina et al., 2018). The primers used for qPCR are listed in Table S4.

The amount of *F. virguliforme* in infected roots was determined for *PSS30*-transgenic soybean plants and non-transgenic Williams

82. Seeds were sown in soil containing *F. virguliforme* isolate NE305S inoculum in a growth chamber. The control plants were grown in uninfected soil. Root tissues were harvested after 21 days following planting of seeds. The relative fungal biomass was calculated by genomic qPCR as amplification of the fungal genomic DNA for the *FvTox1* gene relative to that of the soybean gene *Glyma.05G014200* as an internal control (Brar et al., 2011; Mbofung et al., 2011; Ngaki et al., 2016). DNA (20 ng μl^{-1}) was run in an iQ5 BioRad instrument using SYBR Green qPCR Master Mix. The fold change of the *FvTox1* gene amplification was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Folate measurements

Flg22-treated or *P. sojae*-infected Arabidopsis leaves, *P. sojae*-infected etiolated soybean hypocotyls and *F. virguliforme*- and SCN-infected roots of soybean plants were harvested along with control uninfected tissues, frozen in liquid nitrogen and stored at −80°C until use. Folate contents of individual tissue samples were determined by a microbiological assay using *Lactobacillus rhamnosus* ATCC7469 (American Type Culture Collection, Manassas, VA, USA) as described earlier (Gambonnet et al., 2001). The bioassay is briefly described below.

From each sample, 1 g of tissue powder was considered for determining the level of folate. Total soluble content of the tissues was extracted by grinding the tissues with cold extraction buffer (50 mM phosphate buffer pH 7.0, 50 mM β-mercaptoethanol and 50 mM sodium ascorbate). For folate determination, the clear extract from each sample was boiled and incubated with rat serum that has conjugase activity to remove the polyglutamate tail from the folates. The sample extracts were then added to the double-strength 'folic acid casei' medium and sterilized by autoclaving. After cooling, the *L. rhamnosus* bacterial culture was uniformly added under sterile conditions to each sample extract and incubated at 37°C under shaking for 30 h. After the incubation period, the growth of the bacteria in each sample was measured by measuring the optical density (OD) at 600 nm in a spectrophotometer. In the same assay, a standard curve was developed by using various amounts of pure tetrahydrofolate for studying the growth of the bacteria versus folate content. The amount of folate in each tissue sample was calculated by plotting the OD against the standard folate curve (Gambonnet et al., 2001).

Development of *AtFOLT1*-transgenic plants

The full-length *AtFOLT1* coding sequence was amplified using *Pfu* DNA polymerase from the Col-0 ecotype cDNAs using gene-specific primers (Table S4). The *AtFOLT1* cDNA was first cloned into the pGEM®-T vector (Promega) and sequenced to confirm its identity. The cDNA was then sub-cloned into modified binary pTF102 vectors carrying Prom1 or Prom2. Prom1 is a soybean infection-inducible promoter (*Glyma18g47390*). Prom2 is a soybean root-specific promoter (*Glyma10g31210*) (Ngaki et al., 2016, 2020). For constitutive expression of *AtFOLT1*, the coding region of *AtFOLT1* was cloned into the pISU-Agron5 vector under the control of the 2× CaMV 35S promoter. The three constructs (Prom1-*AtFOLT1*, Prom2-*AtFOLT1* and 35S-*AtFOLT1*) (Figure 3a) were transformed individually into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method.

For the development of transgenic soybean plants, an *A. tumefaciens* isolate carrying pTF102-Prom1-*AtFOLT1*, pTF102-Prom2-*AtFOLT1* or pISU-Agron5-35S-*AtFOLT1* was used to transform soybean cv. Williams 82 at the Plant Transformation Facility, Iowa State University, Ames, IA, USA (Paz et al., 2004). Initially, putative transformants were screened for the expression of the *bar* gene by applying a freshly prepared aqueous solution of 0.2% Liberty (AgrEvo USA, Pikeville, NC, USA) to the mid lamina portion of the second or third youngest leaf. The painted area was marked and responses of the treated tissues were recorded 5–7 days after Liberty application. Putative transformants were confirmed by PCR with *bar*- and *AtFOLT1*-specific primers.

Complementation assay

For the complementation test, the pISU-Agron5 vector carrying 35S-*AtFOLT1* was transformed into the Arabidopsis T-DNA insertion *atfolt1* mutant using a floral dip transformation method (Clough and Bent, 1998). T_1 seeds were harvested from transformed plants and germinated in soil. Transformants were selected by spraying twice with BASTA (60 $\mu g\ ml^{-1}$) at a 4-day interval. T_2 plants were inoculated with *P. sojae* spores and RT-PCR analysis was conducted to confirm expression of the transgene.

The F_1 hybrid (*pss30/atfolt1*) was confirmed by PCR and digestion of the PCR product. For identification of *atfolt1* (T-DNA insertion mutant), PCR was performed using two gene-specific primers and one T-DNA border primer. For *pss30* (EMS mutant), PCR was performed using SBP primers (*pss30*SBP) and PCR-amplified DNA was cut with restriction endonuclease *Rsa*I, which distinguishes mutants from wild type. The phenotypes of the F_1s were characterized and photographed. The primers used for screening of the F_1 hybrids are listed in Table S4.

For folate treatment, each 3-week-old Arabidopsis plant was soil-drenched with 20 ml folic acid solution 48 h before inoculation with *P. sojae* spores (Sumit et al., 2012). The folic acid solutions in water contained 0, 1, 5 or 10 mM folic acid.

Evaluation of *AtFOLT1*-transgenic soybean lines

Transgenic soybean R_1 and R_2 generations were evaluated for responses to *F. virguliforme* under growth chamber and field conditions (Li et al., 2009).

For growth chamber assays, three soybean seeds were planted in a 2:1 mixture of sand and soil mixed with the *F. virguliforme* Mont-1 inocula, prepared on sorghum grains (see the Pathogens and inoculation subsection) at a ratio of 19:1 soil mixture:inocula in 240-ml cups (Dart Container Corporation, Mason, MI, USA). The inoculum was prepared in bulk, mixed thoroughly and used in multiple experiments to reduce experimental variations. Ten replications were considered for each soybean line. The cups were placed in a growth chamber in a completely randomized design and watered once daily. The seedlings were grown at 23°C for 16 h under light (200 μmol photons m^{-2} sec^{-1}) and 16°C for 8 h under dark conditions. Foliar symptoms were scored at 3 and 4 weeks following planting in a 1–7 scale (Paz et al., 2004). Plants were considered resistant if they showed symptoms of score 2 or less; a score of 1 indicates no symptoms and a score of 2 indicates slight yellowing. Plants with scores greater than 2 were considered susceptible (Ngaki et al., 2016; Wang et al., 2018). Data are presented as the mean ± SE of disease scores from 10 replications.

The field trials were conducted from June 11 to October 30 in 2015 and from June 7 to October 30 in 2016 at the Hinds Research Farm, located four miles North of Ames, IA, USA, in a completely randomized block design with three replications. The soybean lines were artificially inoculated with *F. virguliforme* inocula. Twenty-five to 50 seeds of each independent transgenic line were mixed with approximately 15 ml *F. virguliforme* NE305S inocula and sown in a 3-foot single row plot using a hand-push planter. To check BASTA resistance, transgenic lines were sprayed with

glufosinate herbicide (250 mg L^{-1} mixed with 0.1% Tween 20) twice with a 3-day interval at the unifoliate stage. The field was heavily irrigated to generate favorable conditions for SDS symptom development. Individual plants were scored for foliar SDS symptoms at soybean stage R6 using a scale of 1–7, scored as follows: 1, no symptoms; 2, slight symptoms with mottling and mosaic on leaves (1–20% foliage affected); 3, moderate symptoms with interveinal chlorosis and necrosis on foliage (21–50% foliage affected); 4, heavy symptoms with interveinal chlorosis and necrosis (51–80% foliage affected); 5, severe interveinal chlorosis and necrosis (81–100% foliage affected); 6, whole leaf necrosis; and 7, death of plants (Hartman et al., 1997).

Evaluation of *AtFOLT1*-transgenic soybean lines for possible SCN resistance

Two seeds from each soybean line were planted in individual containers filled with soil infested with approximately 50 SCN HG type 2.5.7 cysts (see the Pathogens and inoculation subsection). Each soybean line was planted in 10 containers. The containers of individual lines were placed in a bucket by following a completely randomized design with one or more replications of each genotype in individual buckets. Each bucket can accommodate 18 containers. Buckets with containers were placed in a water bath maintained at 27 $\pm$ 1°C in a greenhouse. Plants in the containers were watered once a day. Thirty days after planting, individual plants were gently pulled out from the container and cysts were gently removed from the roots by washing with high-pressure tap water. Washing was performed on nested 20-mesh sieves (850-μm pores) placed over 60-mesh sieves (250-μm pores) so that the washed cysts were collected over the 60-mesh sieves. The cysts were stored in a small beaker in water and the cysts were counted under a microscope. The female index (FI) was calculated to evaluate the responses of individual genotypes to SCN (Schmitt and Shannon, 1992) as follows:

$$\text{FI}(\%) = \frac{\text{Mean number of cysts on roots of a genotype}}{\text{Mean number of cysts on roots of Williams 82}} \times 100\%$$

Statistical analysis

Statistical significance between genotypes or treatments was determined by conducting the Student *t*-test with the aid of R software (version 3.1.0).

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AUTHOR CONTRIBUTIONS

MKB conceived the project, designed experiments and supervised the project; RS identified the *pss30* mutant; BBS and RS characterized the *pss30* mutant; BBS, RS and MKR conducted genetic mapping of the *PSS30* gene; BBS and RS identified the *PSS30* gene through genome sequencing and T-DNA insertion mutant analyses of candidate *PSS30* genes; BBS and PS conducted complementation analyses; PS and SK identified and analyzed the folate biosynthesis mutants; PS and SK conducted folate-feeding experiments; MNN developed binary plasmid vectors for soybean transformation; MNN also made crosses, evaluated F_1s, conducted qPCR of pathogen genes, maintained transgenic lines in the greenhouse, performed *F. virguliforme* infection assays in growth chambers and conducted RT-PCR for infected transgenic soybean plants; SK, MNN, SS and DRK conducted field trails to evaluate the responses of transgenic soybean plants to *F. virguliforme*; SK, SS and DRK evaluated the responses of transgenic soybean plants to SCN. SS and DRK determined folate contents of transgenic soybean plants infected with SCN and *F. virguliforme*. SK determined folate contents of Arabidopsis and soybean following *P. sojae* infection. SK, BBS, RS, SS, MNN, DRK and PS analyzed the data. SK wrote the first draft of the manuscript. MKB edited and finalized the manuscript. All authors reviewed, commented on and approved the manuscript.

CONFLICT OF INTEREST

All authors claim no conflicts of interest exist.

DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are available upon request.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The *pss30* mutant is susceptible to *P. sojae*.

Figure S2. Molecular mapping of the *PSS30* region.

Figure S3. Genotyping of *pss30* and *atfolt1* T-DNA mutant lines and F_1 (*atfolt1/pss30*) plants.

Figure S4. Molecular characterization of T-DNA insertion mutants for two genes of the folate biosynthesis pathway.

Figure S5. Responses of T-DNA insertion mutants of folate biosynthesis pathway genes to *P. sojae* infection.

Figure S6. Constructs used to generate the transgenic soybean plants. P35S, CaMV 35S promoter; 35S T, CaMV 35S polyA signal; Prom1, promoter 1; Prom2, promoter 2 (for details see the Experimental procedures section).

Figure S7. Senescence of Williams 82 transgenic lines carrying the 35S-*PSS30* transgene.

Figure S8. Transgenic lines carrying the *35S-PSS30* transgene exhibited significantly lower plant height with no effect on yield.

Figure S9. Rapid induction of folate levels in ETI of etiolated soybean hypocotyls following *P. sojae* infection.

Table S1. Segregation of *PSS30* alleles among the $F_{2:3}$ families of a cross between the *pss30* mutant and Nd-0 (*PSS30*).

Table S2. T-DNA insertion mutants of the candidate *PSS30* genes.

Table S3. T-DNA insertion mutants of the genes encoding enzymes of the folate biosynthesis pathway.

Table S4. Primers used in this study.

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