

Overexpression of a soybean salicylic acid methyltransferase gene confers resistance to soybean cyst nematode

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Summary

Salicylic acid plays a critical role in activating plant defence responses after pathogen attack.

Salicylic acid methyltransferase (SAMT) modulates the level of salicylic acid by converting salicylic acid to methyl salicylate. Here, we report that a SAMT gene from soybean (*GmSAMT1*) plays a role in soybean defence against soybean cyst nematode (*Heterodera glycines* Ichinohe, SCN).

GmSAMT1 was identified as a candidate SCN defence-related gene in our previous analysis of soybean defence against SCN using GeneChip microarray experiments. The current study started with the isolation of the full-length cDNAs of *GmSAMT1* from a SCN-resistant soybean line and from a SCN-susceptible soybean line. The two cDNAs encode proteins of identical sequences.

The *GmSAMT1* cDNA was expressed in *Escherichia coli*. Using *in vitro* enzyme assays, *E. coli*-expressed *GmSAMT1* was confirmed to function as salicylic acid methyltransferase. The apparent Km value of *GmSAMT1* for salicylic acid was approximately 46 µM. To determine the role of *GmSAMT1* in soybean defence against SCN, transgenic hairy roots overexpressing *GmSAMT1* were produced and tested for SCN resistance. Overexpression of *GmSAMT1* in

SCN-susceptible backgrounds significantly reduced the development of SCN, indicating that overexpression of *GmSAMT1* in the transgenic hairy root system could confer resistance to SCN. Overexpression of *GmSAMT1* in transgenic hairy roots was also found to affect the expression of selected genes involved in salicylic acid biosynthesis and salicylic acid signal transduction.

Keywords: methyl salicylate, transgenic hairy roots, soybean, *Heterodera glycines*.

Introduction

Heterodera glycines Ichinohe, commonly known as soybean cyst nematode (SCN), is the most economically important pathogen of soybean (*Glycine max* L. Merr.), causing more than \$1 billion in yield losses annually in the United States alone (Koenning and Wrather, 2010). The deployment of SCN-resistant soybean cultivars has been the chief strategy for the management of this pathogen (Rao-Areli *et al.*, 1997). In studying host plant resistance, genetic analysis has led to the identification of a number of major quantitative trait loci for SCN resistance, including *rhg1*, *rhg2*, *rhg3* (Caldwell *et al.*, 1960), *Rhg4* (Matson and Williams, 1965) and *Rhg5* (Rao-Areli *et al.*, 1992). Very recently, exciting progress has been made in gene isolation and functional characterization for *rhg1* and *Rhg4*. Comparative genomic DNA sequencing analysis of SCN-resistant and susceptible lines of soybean combined with transgenic studies has revealed that *rhg1-b*-mediated nematode resistance is determined by the copy number variation of multiple genes at *Rhg1* (Cook *et al.*, 2012). In another study, a serine hydroxymethyltransferase (SHMT) gene at the *Rhg4* locus has been isolated using map-based cloning (Liu *et al.*, 2012). The alteration of a key regulatory property of SHMT due to genetic polymorphisms was found to determine SCN resistance and susceptibility. The distinct

resistance mechanisms conferred by *rhg1* and *Rhg4* show the complexity of SCN resistance.

In addition to QTL analysis and positional cloning of resistance genes, a number of microarray studies have been performed to identify candidate genes for SCN resistance from soybean and to help better understand the mechanisms of resistance (Alkharouf *et al.*, 2006; Ithal *et al.*, 2007; Kandoth *et al.*, 2011; Khan *et al.*, 2004; Klink *et al.*, 2007, 2009, 2010, 2011; Lu *et al.*, 2006; Matsye *et al.*, 2011; Mazarei *et al.*, 2011; Puthoff *et al.*, 2007). A subset of these studies analysed differential gene expression patterns of a single soybean cultivar during the compatible and incompatible interactions with SCN; another subset of microarray studies compared two soybean lines against the same race of SCN population; a final subset of studies analysed the time to rapid and potent localized resistant reactions at the syncytia using a variety of soybean germplasm. Previously, we used the Affymetrix soybean GeneChip to study SCN infection in two soybean lines: TN02-226 and TN02-275, which are resistant and susceptible to SCN race 2 (HG type 1.2.5.7), respectively (Mazarei *et al.*, 2011). TN02-226 and TN02-275 are two F₆-derived sister lines that were developed as recombinant inbred lines from hybridizing SCN-resistant parents 'Fowler' and 'Anand'. This investigation led to the identification of a large number of differentially expressed genes between the two soybean lines. Upon SCN infection, the

expression levels of 162 and 1,694 transcripts changed significantly in the resistant and susceptible lines, respectively (Mazarei et al., 2011). Based on the functional categories of the identified putative defence genes, this and other microarray studies suggested that SCN resistance of soybean might involve complex signalling pathways, one of which is the salicylic acid (SA) pathway (Klink et al., 2007, 2010; Mazarei et al., 2011).

The role of the SA signalling pathway in plant defence against pathogens, especially bacteria (Sanchez-Rojo et al., 2011) and viruses (Park et al., 2007), has been well characterized. Recent studies suggest that this pathway also has a role in plant defence against nematodes. The role of the SA pathway in resistance to parasitism by nematodes was first suggested by examining the effect of exogenously applied SA on plant-parasitic nematode reproduction. For example, treatment of white clover roots with SA prior to inoculation led to the decreased reproduction of the clover cyst nematode (*Heterodera trifolii*) (Kempster et al., 2001). There is also evidence that nematodes secrete chorismate mutase that modulates the levels of SA in the infested plant (Doyle and Lambert, 2003). In *Arabidopsis thaliana*, the study provided genetic evidence about the role of the SA pathway in nematode resistance. SA-deficient mutants exhibited increased susceptibility to beet cyst nematode (*Heterodera schachtii*). Nonexpressor of pathogenesis-related genes 1 (*NPR1*) is a central component of the SA signal transduction pathway (Pieterse and Van Loon, 2004). *A. thaliana* plants containing a mutant *NPR1* gene showed increased susceptibility to *H. schachtii* (Wubben et al., 2008).

One of the SA pathway genes to be identified in our previous study (Mazarei et al., 2011) was *Glyma02g06070*, which was annotated to encode a protein that is highly homologous to S-adenosyl-L-methionine (SAM)-dependent salicylic acid carboxyl methyltransferases (SAMTs). *Glyma02g06070* was designated as *GmSAMT1* (*Glycine max* salicylic acid methyltransferase gene 1). The expression of *GmSAMT1* in TN02-226, the resistant soybean line, was significantly induced at 3, 6 and 9 days postinoculation (dpi) with SCN. In contrast, its expression in TN02-275, the susceptible line, was not significantly changed by SCN infection (Mazarei et al., 2011). As a component of the SA pathway, SAMT catalyses the methylation of SA using SAM as the methyl donor to form methyl salicylate (MeSA). In tobacco, MeSA was demonstrated to be the mobile signal-mediating systemic acquired resistance (Park et al., 2007). Prior to this study, SAMT genes have been isolated and characterized from a number of plant species, including *Clarkia breweri* (Ross et al., 1999), snapdragon (*Antirrhinum majus*) (Negre et al., 2002), *Stephanotis floribunda* (Pott et al., 2002), *Nicotiana suaveolens* (Pott et al., 2004), *Arabidopsis* (Chen et al., 2003a), rice (Koo et al., 2007; Zhao et al., 2010), tobacco (Park et al., 2007) and tomato (Tieman et al., 2010). The SAMT genes from *Arabidopsis*, rice and tobacco have been shown to be involved in plant defence. Based on this knowledge, as well as the differential expression pattern of *GmSAMT1* in resistant and susceptible soybean lines, we hypothesized that *GmSAMT1* plays a role in SCN resistance by modulating the SA signalling pathway.

Here, we report the detailed functional characterization of *GmSAMT1*. The biochemical activity of the enzyme encoded by *GmSAMT1* was characterized using *Escherichia coli*-expressed recombinant *GmSAMT1*. The biotechnological use of *GmSAMT1* in mediating soybean defence against SCN was assessed using transgenic hairy roots overexpressing *GmSAMT1*. The possible mechanistic aspects of SCN resistance conferred by *GmSAMT1*

were studied by analysing the expression of selected genes in the SA biosynthetic and signal transduction pathways.

Results

Cloning and sequence analysis of *GmSAMT1*

Full-length cDNAs for the two alleles of *GmSAMT1* in TN02-226 and TN02-275 were cloned and fully sequenced. The inferred sequences of the proteins encoded by these two alleles were identical; they were also identical to the protein sequence of *GmSAMT1* from 'Williams 82', the cultivar used for whole-genome sequencing (Schmutz et al., 2010). *GmSAMT1* showed the highest level of sequence similarity to the SAMT from *C. breweri* (CbSAMT) with a 58% identity among characterized SAMTs. In addition, most amino acid residues in the active site of *GmSAMT1* are conserved compared to those of CbSAMT and other known SAMTs (Figure 1). SAMTs belong to the SABATH family of methyltransferases, which include other methyltransferases, such as jasmonic acid methyltransferase (JAMT) (Seo et al., 2001), indole-3-acetic acid methyltransferase (IAMT) (Qin et al., 2005; Zhao et al., 2007, 2008) and gibberellic acid methyltransferase (GAMT) (Varbanova et al., 2007). Phylogenetic analysis showed that *GmSAMT1* is more closely related to the known SAMTs than other types of methyltransferases (Figure 2).

Biochemical properties of *GmSAMT1*

Recombinant *GmSAMT1* expressed in *Escherichia coli* was purified to electrophoretic homogeneity and tested with a number of carboxylic acids as potential substrates. Of substrates tested, *GmSAMT1* showed the highest level of specific activity with SA. It could also use benzoic acid, anthranilic acid and 3-hydroxy benzoic acid as substrates, with specific activities of 16.9%, 9.3% and 1.8% of that with SA, respectively. Other carboxylic acids, including jasmonic acid, indole-3-acetic acid and gibberellic acid, could not serve as substrate for *GmSAMT1*.

The biochemical properties of *GmSAMT1* using SA as substrate were analysed in detail. When enzyme assays were performed in buffers of different pH values, *GmSAMT1* showed the highest level of catalytic activity at pH 7.5 (Figure 3a). The optimal temperature for *GmSAMT1* activity was 25 °C (Figure 3b). The effects of various cations on *GmSAMT1* activity were measured. Compared with control, *GmSAMT1* exhibited a twofold increase in activity in the presence of K⁺, while its activity was quite inhibited by Cu²⁺, Zn²⁺, Fe²⁺ and Fe³⁺. *GmSAMT1* activity was moderately inhibited by Mn²⁺ and Ca²⁺. Other ions, including Na⁺, NH₄⁺ and Mg²⁺, had no effect on *GmSAMT1* activity (Figure 3c). Increases in reaction rate by increasing concentrations of SA were found to obey the Michaelis–Menten kinetics. *GmSAMT1* catalysed the methylation of SA with an apparent K_m value of 46.2 ± 4.2 μM for SA (Figure 3d).

Production of transgenic hairy roots overexpressing *GmSAMT1*

A transgenic hairy root system was used to assay whether overexpression of *GmSAMT1* in soybean roots could be useful to confer resistance against SCN. To perform vigorous SCN bioassays, it is vital to nondestructively identify transgenic hairy roots overexpressing *GmSAMT1*. To this end, we chose to coexpress *GmSAMT1* with an orange fluorescent protein (OFP) marker gene (Alieva et al., 2008; Mann et al., 2012). The effectiveness of coexpression in transgenic hairy roots was validated using OFP

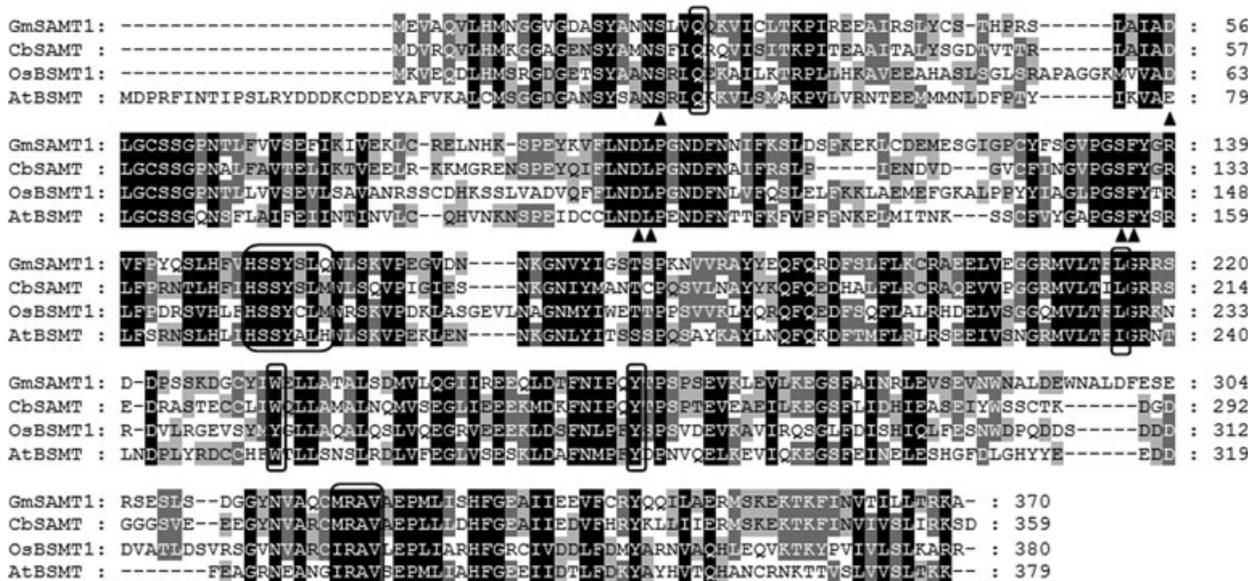
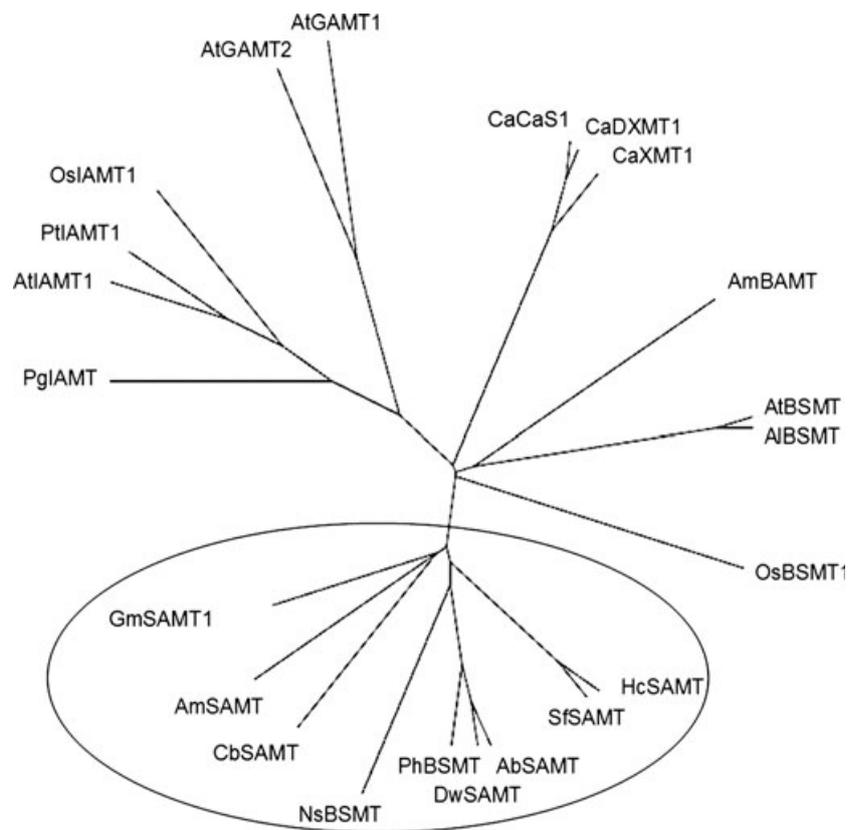


Figure 1 Multiple sequence alignment of GmSAMT1 with selected known SAMTs. The triangles indicate the amino acids for binding of the cofactor S-adenosyl-L-methionine (SAM). The rectangles represent the amino acids for substrate binding. CbSAMT, *Clarkia breweri* SAMT (AF133053); OsBSMT1, *Oryza sativa* BSMT1 (XM467504); AtBSMT, *Arabidopsis thaliana* BSMT (BT022049).

Figure 2 A phylogenetic tree containing GmSAMT1 and other selected functionally characterized members of the plant SABATH family of methyltransferases. CbSAMT, *Clarkia breweri* SAMT (AF133053); AmSAMT, *Antirrhinum majus* SAMT (AF515284); SfsSAMT, *Stephanotis floribunda* SAMT (AJ308570); HcSAMT, *Hoya carnososa* SAMT (AJ863118); DwSAMT, *Datura wrightii* SAMT (EF472972); AmBAMT, *Antirrhinum majus* BAMT (AF198492); NsBSMT, *Nicotiana suaveolens* BSMT (AJ628349); AtBSMT, *Arabidopsis thaliana* BSMT (BT022049); AIBSMT, *A. lyrata* BSMT (AY224596); AbSAMT, *Atropa belladonna* SAMT (AB049752); OsBSMT1, *Oryza sativa* BSMT1 (XM467504); PhBSMT, *Petunia hybrida* BSMT (AY233465); OsiAMT1, *O. sativa* IAMT1 (EU375746); PtiAMT1, *Populus trichocarpa* IAMT1(XP_002298843); AtIAMT, *A. thaliana* IAMT (AK175586); AtGAMT1, *A. thaliana* GAMT1 (At4 g26420); AtGAMT2, *A. thaliana* GAMT2 (At5 g56300); CaCaS1, *Coffea arabica* caffeine synthase 1 (AB086414); CaXMT1, *C. arabica* XMT1 (AB048793); CaDXMT1, *C. arabica* DXMT1 (AB084125).



and the β -glucuronidase (GUS) gene (Figure S1). We constructed two new binary vectors with one containing two cassettes for overexpressing *GmSAMT1* and *OFF*, respectively (Figure 4a), and the other containing only the *OFF* cassette (Figure 4b). The vector with *GmSAMT1-OFF* two cassettes was used to produce transgenic hairy roots in two soybean lines: the breeding line TN02-

275 and cv 'Williams 82', both of which are susceptible to SCN race 2. The vector with only the *OFF* cassette was used to produce control transgenic hairy roots in three soybean lines: TN02-275, 'Williams 82' and the SCN-resistant breeding line TN02-226. No apparent differences in the efficiency of hairy root generation among the five types of transgenic hairy roots were observed.

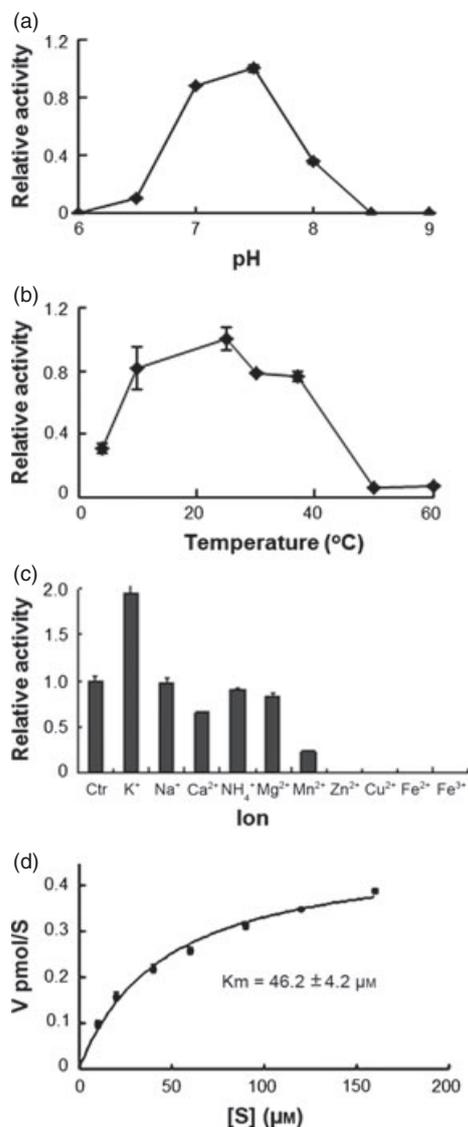


Figure 3 Biochemical properties of GmSAMT1. (a) The pH effect on GmSAMT1 activity. Level of GmSAMT1 activity in 50 mM Tris-HCl buffer, pH 7.5, was arbitrarily set at 1.0. (b) Optimal temperature of GmSAMT1. The level of GmSAMT1 activity at 25°C was arbitrarily set at 1.0. (c) Effects of cations on activity of GmSAMT1. Cations were independently added to reactions in the form of chloride salts at 5 mM final concentrations. The level of GmSAMT1 activity without adding the tested cations as control (Ctr) was arbitrarily set at 1.0. (d) GmSAMT1 displayed pseudo Michaelis–Menten kinetics, with apparent Km value of $46.2 \pm 4.2 \mu\text{M}$ for salicylic acid.

Approximately 70% of the *A. rhizogenes*-infected soybean plants produced transgenic hairy roots. The expression levels of *GmSAMT1* in the five types of transgenic hairy roots, which were initially selected by the presence of OFF signal, were determined using qRT-PCR. In non*GmSAMT1*, OFF-overexpressing transgenic hairy roots, there was no difference in *GmSAMT1* transcript abundance between the SCN-resistant and SCN-susceptible backgrounds (Figure 4c). In contrast, the transcript abundance of *GmSAMT1* in the two lines of *GmSAMT1*-overexpressing transgenic hairy roots was more than 20 times higher than those in their respective control hairy roots (Figure 4c).

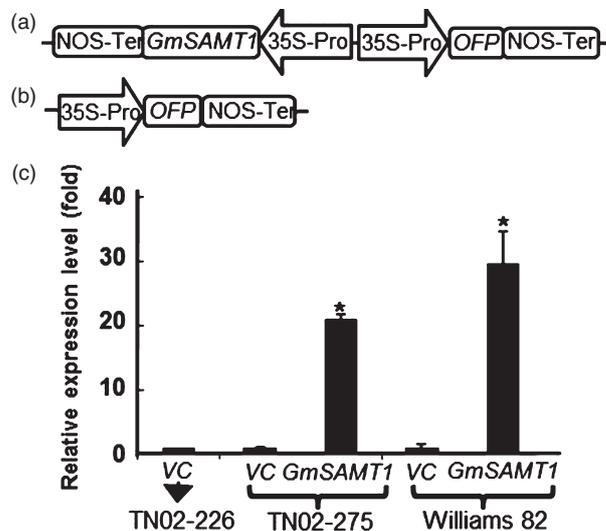


Figure 4 Production of transgenic hairy roots of soybean overexpressing *GmSAMT1*. (a) Schematic representation of the construct used for coexpression of an orange fluorescent protein (OFF) reporter gene and *GmSAMT1*. '35S-Pro' and 'NOS-ter' represent the CaMV 35S promoter and the NOS terminator, respectively. (b) Schematic representation of the construct used for overexpression of an orange fluorescent protein (OFF) reporter gene. '35S-Pro' and 'NOS-ter' represent the CaMV 35S promoter and the NOS terminator, respectively. (c) Expression of *GmSAMT1* in five types of transgenic hairy roots from three genetic backgrounds TN02-226, TN02-275 and 'Williams 82'. 'GmSAMT1' represents transgenic hairy roots containing *GmSAMT1* transgene. 'VC' represents transgenic hairy roots produced using a control vector containing OFF marker but not *GmSAMT1* gene. qRT-PCR was performed with *GmSAMT1*-specific primers. Expression values were normalized to the expression levels of the soybean ubiquitin 3 gene (*GmUBI3*) in respective samples. The level of *GmSAMT1* expression in the 'VC' transgenic hairy roots of TN02-275 was arbitrarily set at 1.0. Each bar represents the mean relative expression level of three independent experiments with the standard errors, each containing a pool of five plants. The bars with asterisks are significantly different (LSD $P < 0.01$).

Evaluation of SCN development in transgenic hairy roots overexpressing *GmSAMT1*

We examined the effectiveness of *GmSAMT1* overexpression on SCN resistance using the hairy root assay. Five types of transgenic hairy roots, which comprised overexpression of *GmSAMT1* in 'Williams 82', 'Williams 82'-vector control, overexpression of *GmSAMT1* in TN02-275, TN02-275-vector control and TN02-226-vector control, were subject to infection with SCN race 2. Two weeks after inoculation with SCN eggs, transgenic hairy roots were evaluated for SCN development following a recently established protocol by measuring the ratio of the nematodes developed beyond J2 stage to the total number of penetrating nematodes (Cook *et al.*, 2012; Melito *et al.*, 2010). Female nematodes from J2 to J4 or adult stage were observed for most sections of transgenic hairy roots, and male nematodes were occasionally observed (Figure 5a). The mean number of total nematodes for each type of hairy root was from 16.3 to 26.5, and the mean number of nematodes beyond J2 stage was from 0.7 to 4.1. There was no significant difference on the total number of nematodes among transgenic and control lines, while there was a significant difference on the nematodes developed beyond J2

stage (J3+ J4+ adult). Therefore, the normalized ratio of the number of SCNs that had developed beyond J2 stage (J3+ J4+ adult) to the total number of nematodes per plant was used as an index to indicate the resistance levels of the transgenic hairy roots harbouring the various constructs (Cook *et al.*, 2012; Melito *et al.*, 2010). The resistance indices in transgenic hairy roots overexpressing *GmSAMT1* relative to 'Williams 82'-vector control and TN02-275-vector control were 46.6% and 27.6%, respectively, which were comparable to 24.3% of TN02-226 vector control (Figure 5b).

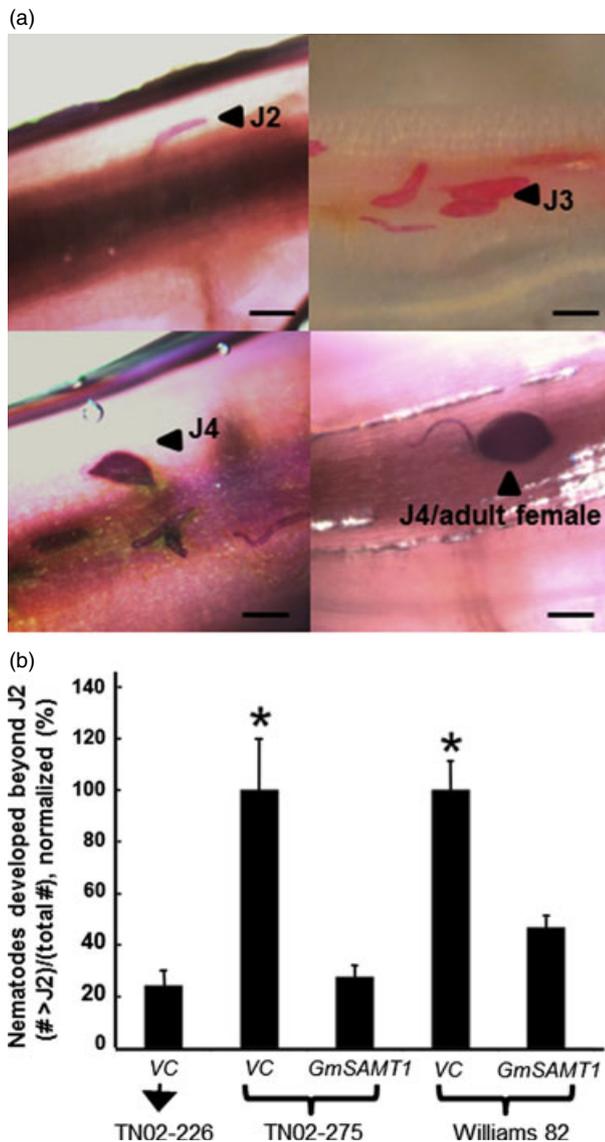


Figure 5 SCN performance in transgenic hairy roots. (a) Representative stages of SCN in the hairy roots. Bars = 250 μ m. (b) Five types of transgenic hairy roots from three genetic backgrounds TN02-226, TN02-275 and 'Williams 82' were analysed. '*GmSAMT1*' represents transgenic hairy roots containing the *GmSAMT1* transgene. 'VC' represents transgenic hairy roots produced using a control vector containing GFP marker but not *GmSAMT1*. Each bar represents the mean of resistance index with the standard errors, the normalized ratio of the nematodes developed beyond J2 stage to total number of nematodes per plant. Bars with asterisks are significantly different (LSD $P < 0.01$).

Expression of SA biosynthetic and signal transduction genes in transgenic hairy roots overexpressing *GmSAMT1*

To understand the possible mechanism underlying *GmSAMT1*-mediated resistance to SCN, we analysed the expression of selected SA biosynthetic and signal transduction genes in *GmSAMT1*-transgenic hairy roots. Isochorismate synthase (ICS) is critical for pathogen-induced SA biosynthesis (Garcion *et al.*, 2008). There are two ICS homologs in soybean, *GmICS1* (*Glyma01g25690*) and *GmICS2* (*Glyma03g17420*). Without SCN treatment, both *GmICS1* and *GmICS2* showed higher transcript abundance (5.3 times and 8.2 times) in the *GmSAMT1*-transgenic hairy roots than those in the control hairy roots (Figure 6). In SCN-inoculated hairy roots, *GmICS1* showed similar levels of expression in *GmSAMT1*-transgenic hairy roots and the control hairy roots, while *GmICS2* had higher expression in *GmSAMT1*-transgenic hairy roots than that in the control hairy roots. When the expression levels of the two genes were compared in the same sample with and without SCN treatment, both *GmICS1* and *GmICS2* were found to be significantly up-regulated by SCN treatment in control hairy roots. In *GmSAMT1*-transgenic hairy roots, both genes showed similar levels of expression between SCN-treated and untreated samples (Figure 6).

NPR1 is a component of the SA signalling pathway, and it is central to the activation of SA-dependent defence genes (Pieterse and Van Loon, 2004). To determine whether overexpression of *GmSAMT1* has an impact on the SA signal transduction pathway, the expression of two NPR1 homologs in soybean, *GmNPR1-1* (*Glyma09g02430*) and *GmNPR1-2* (*Glyma15g13320*) (Sandhu *et al.*, 2009), was analysed (Figure 7). Without SCN treatment, the expression of *GmNPR1-1* in the *GmSAMT1*-transgenic hairy roots was similar to that in the control hairy roots, whereas *GmNPR1-2* showed higher transcript abundance in the *GmSAMT1*-transgenic hairy roots than that in the control hairy roots (Figure 7). Upon SCN treatment, the expression of *GmNPR1-1* was higher in the *GmSAMT1*-transgenic hairy roots than that in the control hairy roots, but the expression of *GmNPR1-2* remained unchanged. When the expression levels of the two genes were compared in the same sample, SCN treatment was found to have no significant effect on the expression of either gene (Figure 7).

Discussion

Elucidating the defence mechanisms of soybean against SCN provides a valuable means for development of novel SCN-resistant soybean varieties. Large-scale gene expression analysis has led to the identification of a large number of genes that are putatively involved in the resistance/susceptibility of soybean to SCN (Kandathil *et al.*, 2011; Khan *et al.*, 2004; Klink *et al.*, 2007, 2009, 2010, 2011; Lu *et al.*, 2006; Matsye *et al.*, 2011; Mazarei *et al.*, 2011; Puthoff *et al.*, 2007). The functions of these genes, however, cannot be solely determined based on gene expression analysis due to the complexity of the interactions between soybean and SCN. Recently, Matthews *et al.* (2013) performed functional analysis of a group of soybean genes selected from published microarray studies by individually overexpressing these genes in hairy roots. From the 100 genes analysed, about 10% were found to be associated with at least a 50% reduction of female SCN. About 17% of the genes increased the number of female nematodes by more than 150%, and about half the genes had no significant effect on SCN development. That study demon-

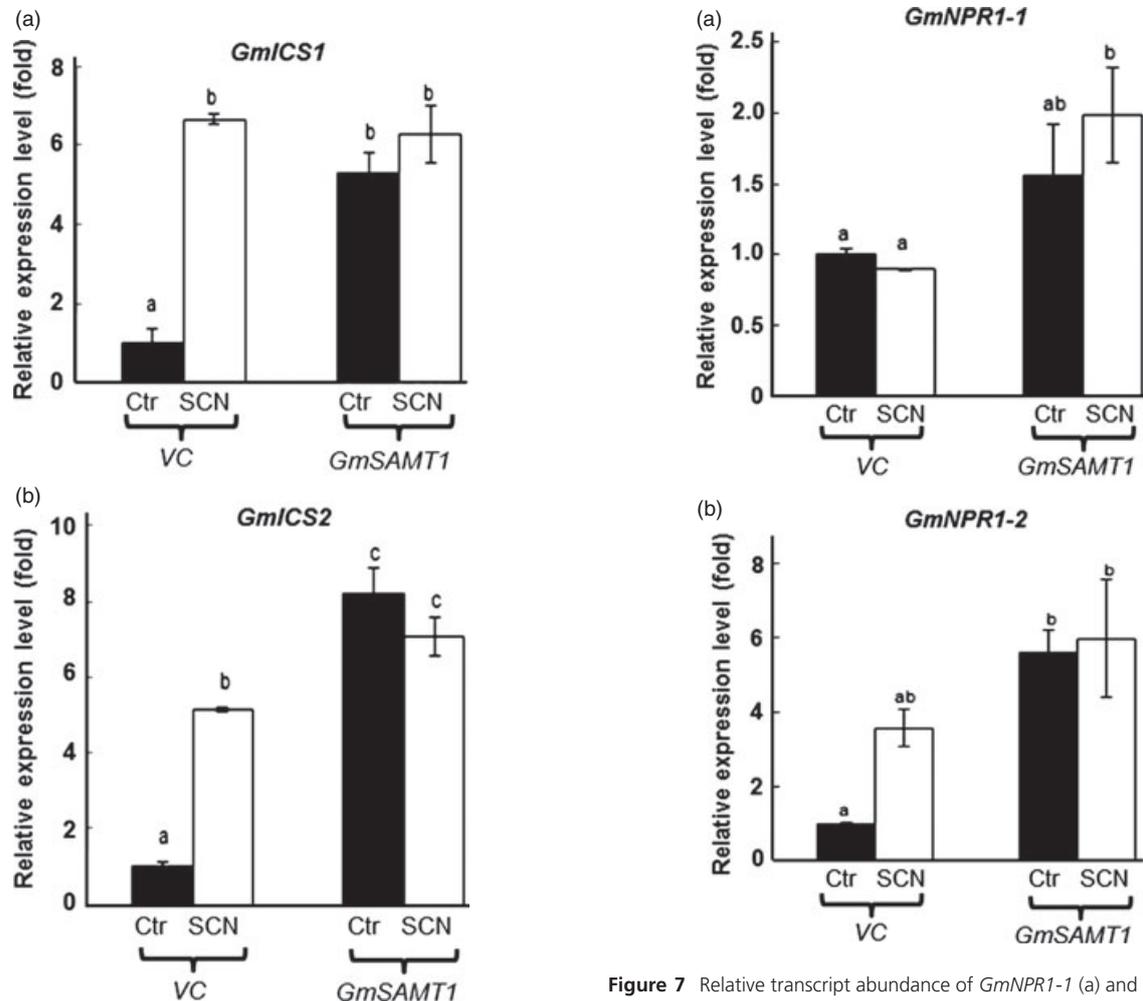


Figure 6 Relative transcript abundance of *GmICS1* (a) and *GmICS2* (b) in transgenic hairy roots with (SCN) or without (Ctr) SCN infection. Transgenic hairy roots containing the *GmSMT1* transgene (*GmSMT1*) and transgenic hairy roots containing an orange fluorescent protein reporter gene but not *GmSMT1* (VC) were produced in the 'Williams 82' background and analysed. qRT-PCR was performed with *GmSMT1*-specific primers. Expression values were normalized to the expression levels of the soybean ubiquitin 3 gene (*GmUBI3*) in respective samples. The relative transcript abundance of *GmSMT1* in the 'VC' transgenic hairy roots without SCN infection was arbitrarily set at 1.0. Each bar represents the mean relative expression level of three independent experiments with the standard errors, each containing a pool of five plants. Letters indicate significant differences (LSD $P < 0.05$).

strated the necessity of functional validation of putative soybean defence genes in SCN resistance through *in planta* studies.

The present study has provided evidence for the *in planta* role of *GmSMT1* in soybean defence against SCN. The normalized ratio of the number of SCNs that had developed beyond J2 stage to the total number of nematodes per plant was significantly lower for *GmSMT1* hairy roots than the controls in both the TN02-275 and 'Williams 82' backgrounds. When the susceptibility levels of the control transgenic hairy roots in the two susceptible soybeans TN02-275 and 'Williams 82' were arbitrarily set at 100%, the transgenic soybean hairy roots of these two soybeans overexpressing *GmSMT1* achieved 72.4% and 53.4% reduction in susceptibility to SCN, respectively. These

Figure 7 Relative transcript abundance of *GmNPR1-1* (a) and *GmNPR1-2* (b) in transgenic hairy roots with (SCN) or without (Ctr) SCN infection. Transgenic hairy roots containing the *GmSMT1* transgene (*GmSMT1*) and transgenic hairy roots containing an orange fluorescent protein reporter gene but not *GmSMT1* (VC) were produced in the 'Williams 82' background and analysed. qRT-PCR was performed with *GmSMT1*-specific primers. Relative transcript abundance of the genes of interest was normalized to that of soybean ubiquitin 3 gene (*GmUBI3*) in respective samples. The relative transcript abundance of *GmSMT1* in the 'VC' transgenic hairy roots without SCN infection ('Ctr') was arbitrarily set at 1.0. Each bar represents the mean relative transcript abundance of three independent experiments with the standard errors, each containing a pool of five plants. Letters indicate significant differences (LSD $P < 0.05$).

results suggest that *GmSMT1* can be an effective gene for improving soybean resistance against SCN in transgenic soybean. For the two pairs of transgenic hairy roots in the background of TN02-275 and 'Williams 82', respectively, despite the significant developmental differences of SCN, there were no differences observed in total SCN between transgenics and controls. Therefore, *GmSMT1* hindered the developmental progression of infecting SCNs in the soybean roots.

The initial identification of *GmSMT1* as a candidate defence-related gene to SCN was based on its expression pattern in SCN-resistant and SCN-susceptible soybean lines (Mazarei *et al.*, 2011). Clearly, resistance is not conferred by allelic differences or biochemical properties, as all *GmSMT1* alleles we observed from multiple lines were monomorphic. Therefore, resistance/susceptibility is associated with the transcript levels of *GmSMT1*; there

were similar levels of expression in the roots of TN02-275 and TN02-226 without SCN infection (Figure 4b). The difference in the levels of *GmSAMT1* expression was detectable at 3, 6 and 9 days postinoculation with SCN (Mazarei *et al.*, 2011). This suggests that the distinct levels of resistance/susceptibility of TN02-275 and TN02-226 soybean lines to SCN are at least partly due to the differences in the levels of *GmSAMT1* expression induced by SCN infection. Interestingly, the promoter sequences of two *GmSAMT1* alleles from the SCN-resistant and susceptible soybean lines are identical (Jingyu Lin, Mitra Mazarei, Charles Neal Stewart and Feng Chen, unpublished). Therefore, higher transcript induction and resistance is likely the product of a unique transcription factor sequence in the resistant line.

The mechanism of how *GmSAMT1* negatively impacted SCN development is unknown. However, the biochemical activity of *GmSAMT1* hints at a possible mechanism. To recap, bacterial-expressed recombinant *GmSAMT1* functions as an S-adenosyl-L-methionine-dependent salicylic acid methyltransferase. It catalyses the formation of MeSA using SA as substrate. The biochemical properties of *GmSAMT1* provide additional evidence that this enzyme activity is biologically relevant. The apparent K_m value of *GmSAMT1* for SA was determined to be approximately $46 \mu\text{M}$ (Figure 3d), which is comparable to the K_m values of known SAMTs from other plants. For example, the K_m values of the rice SAMT and the tomato SAMT using SA substrate were $37.4 \mu\text{M}$ (Zhao *et al.*, 2010) and $50 \mu\text{M}$ (Tiemann *et al.*, 2010), respectively. Therefore, the K_m of *GmSAMT1* is at the range of concentrations of SA that are biologically relevant. While SAMTs have been shown to be involved in multiple biological processes, including floral scent production (Ross *et al.*, 1999), defence against pathogens (Park *et al.*, 2007) and defence against insects (Zhao *et al.*, 2010), *GmSAMT1* is the first SAMT gene to be shown to have a role in defence against nematodes. It remains to be determined whether SAMTs in other plants are also involved in defence against nematodes and other pathogens.

While it remains to be determined which is the mechanism underlying the differential expression of *GmSAMT1* upon SCN infection in resistant and susceptible lines, our gene expression analysis of *GmICSs* and *GmNPR1s* provides additional information about how the induced expression of *GmSAMT1* led to increased resistance to SCN. There were essentially no significant changes in the expression levels of *GmICSs* and *GmNPR1s* in *GmSAMT1*-overexpressing transgenic hairy roots with or without SCN inoculation. However, the expression levels of these genes, except *GmNPR1-1*, in *GmSAMT1*-overexpressing transgenic hairy roots were significantly higher than those in the control hairy roots without SCN infection (Figures 6 and 7). This result suggests that the higher level of resistance of *GmSAMT1*-overexpressing hairy roots is not because of enhanced induced defence upon SCN infection. Instead, the overexpression of *GmSAMT1* may have primed the plants for defence responses through the modulation of both SA biosynthetic and signal transduction pathway prior to pathogen infection.

In summary, we have demonstrated that the SCN defence-related soybean gene *GmSAMT1* encodes an S-adenosyl-L-methionine-dependent salicylic acid methyltransferase in *in vitro* enzyme assays. It uses salicylic acid, a plant immune signal, as a substrate to produce methyl salicylate. Overexpression of *GmSAMT1* appeared to modulate both SA biosynthesis and SA signal transduction, evidenced by the induced expression of *ICS* and *NPR1* genes in *GmSAMT1*-overexpressing transgenic hairy roots. Overexpression of *GmSAMT1* in soybean roots reduced the

susceptibility of susceptible soybeans to nematode infection, indicating that *GmSAMT1* plays a role in soybean defence against SCN. Our next step is to assess the nematode resistance of stably transformed soybean overexpressing *GmSAMT1*. In addition, the effects of *GmSAMT1* overexpression on plant resistance to foliar pathogens and other agronomic traits will be evaluated in stable transgenic soybeans.

Experimental procedures

Plant, nematode, bacterium and chemical sources

Three soybean genotypes were used in this study: TN02-226, TN02-275 and 'Williams 82'. TN02-226 and TN02-275 are two sister breeding lines wherein the former is SCN-resistant (Mazarei *et al.*, 2011). 'Williams 82' was the cultivar used for soybean whole-genome sequencing (Schmutz *et al.*, 2010). SCN race 2 was used for SCN bioassays. The maintenance of SCN followed a previously described method (Arelli *et al.*, 2000). *Escherichia coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA) was used for the expression of recombinant protein. *Agrobacterium rhizogenes* strain K599 was utilized for the generation of the soybean hairy roots. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation of full-length cDNAs of *GmSAMT1*

Total RNA was isolated from the SCN-infected root tissues of soybean using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA), and DNA contamination was removed with an on-column DNase (Qiagen, Valencia, CA) treatment. Then, cDNA was synthesized using the First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ) as previously described (Chen *et al.*, 2003b). Full-length cDNAs of *GmSAMT1* were amplified via PCR using the following two gene specific primers: the forward primer 5'-ATGGAAGTAGCACAGGTACTCCACATG-3' and the reverse primer 5'-TGCTTTTCTAGCAATAATATGGTAAC-3'. The PCR conditions were as follows: 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min 30 s and a final extension at 72 °C for 10 min. PCR products were cloned into the vector pEXP5/CT-TOPO and fully sequenced.

GmSAMT1 was subcloned into a pET100/D-TOPO vector (Invitrogen, Carlsband, CA) and expressed in the *E. coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA). Protein synthesis was induced by isopropyl β -D-1-thiogalactopyranoside at a concentration of $500 \mu\text{M}$ for 18 h at 25 °C, with cells lysed by sonication. *E. coli*-expressed *GmSAMT1* with a His-tag was purified from the *E. coli* cell lysate using Ni-NTA agarose following the manufacturer instructions (Invitrogen). Protein purity was verified by SDS-PAGE, and protein concentrations were determined by the Bradford assay (Bradford, 1976).

Multiple sequence alignment and phylogenetic tree construction

A multiple sequence alignment of *GmSAMT1* with selected known SAMT proteins was performed using ClustalX program. The neighbor-joining unrooted phylogenetic tree was constructed using PAUP3.0 software and viewed using TreeView software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Radiochemical SAMT activity assay

Radiochemical SAMT assays were performed according to Zhao *et al.* (2007). A 50 μL volume containing 50 mM Tris-HCl, pH 7.5,

1 mM SA and 3 μM ^{14}C -SAM with a specific activity of 51.4 mCi/mmol (Perkin-Elmer, Boston, MA) was used. The assay was initiated by addition of SAM, maintained at 25 °C for 30 min and stopped by the addition of ethyl acetate (150 μL). After phase separation by 1 min centrifugation at 14,000 g , the upper organic phase was counted using a liquid scintillation counter (Beckman Coulter, Fullerton, CA) as previously described (D'Auria et al., 2002). The radiation counts in the organic phase indicated the amount of synthesized MeSA. Substrate specificity assay was also performed for recombinant GmSAMT1, with a range of substrates including SA, benzoic acid, anthranilic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, nicotinic acid, p-coumaric acid, caffeic acid, cinnamic acid, vanillic acid, jasmonic acid, gibberellic acid, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid. The activity with SA was set at 100% for relative comparisons. Three independent assays were performed for each compound.

Determination of kinetic parameters of GmSAMT1

Appropriate enzyme concentrations and incubation time were determined in time course assays to make sure that the reaction velocity was linear during the assay period. To determine the K_m value for SA, the concentration of SAM was fixed at the saturated level (200 μM), and the concentration of SA was varied from 10 to 160 μM . Assays were conducted at 25 °C for 30 min, as described in the SAMT activity assay. The kinetic parameter K_m was calculated with GraphPad Prism 5 software for Windows (GraphPad Software Inc.), using standard settings for nonlinear regression curve fitting for the Michaelis–Menten equation. The pH optimum, optimal temperature and cation effectors were determined following the protocol previously published (Zhao et al., 2007).

Construction of binary vectors for root transformation

The pCAMBIA 1305.2 vector was used as a backbone binary vector. The coding sequence of an orange fluorescent protein (OFP) (Mann et al., 2012), which was originally termed *pporRFP* (GenBank accession number DQ206380) (Alieva et al., 2008), was amplified and modified to remove *HindIII* restriction site through PCR-mediated site-directed mutagenesis, then inserted into the *NcoI* site of pCAMBIA 1305.2, which resulted in a vector named pJL-OFP. Next, the cassette of 35S::GUS/NOS terminator was excised from a pBI121 vector by digestion with *EcoRI* and *HindIII* and inserted into the *EcoRI* and *HindIII* sites of pJL-OFP, which led to a new vector named pJL-OFP-35S::GUS. Then vector pJL-OFP-35S::GUS was used to test the effectiveness of using OFP as a marker to identify transgenic lines for the overexpression of the target gene (GUS in this case). The *GmSAMT1* cDNA was inserted into the *BamHI* and *SacI* sites of pJL-OFP-35S::GUS to replace the GUS gene, which resulted in the pJL-OFP-35S::GmSAMT1 construct. The constructs including pJL-OFP, pJL-OFP-35S::GUS and pJL-OFP-35S::GmSAMT1 were introduced into the *A. rhizogenes* strain K599 by the freeze-thaw method (Chen et al., 1994).

Generation of transgenic soybean hairy roots

Soybean seeds were first sterilized with chlorine gas overnight and then transferred to autoclaved filter paper moistened with sterile distilled water for germination. The germinated soybean seeds were sowed in sterile vermiculite, and six seeds were grown in each cell of an 18-cell germination tray. The overnight cultured bacteria lawn of *A. rhizogenes* K599 with binary vector

pJL-OFP, pJL-OFP-35S::GUS or pJL-OFP-35S::GmSAMT1 from an LB medium plate containing 50 mg/mL kanamycin was collected and suspended in 1 mL of sterile distilled water for soybean transformation. Soybean hairy roots were generated as previously reported (Kereszt et al., 2007). The growth conditions for soybean plants were 16 h light/8 h dark at 22 °C with irradiance from cool white fluorescent bulbs at 150–200 $\mu\text{mol m}^{-2}\text{s}^{-1}$. After about 4 weeks, the hairy roots grew to approximately 10 cm in length. Soybean transgenic roots were detected based on OFP expression, using an epifluorescent microscope (Olympus stereo microscope model SZX12, Olympus America, Center Valley, PA) with a tdTomato filter set: 535/30 nm excitation and 600/50 nm band pass emission and QCapture 2.56 imaging software.

The OFP-positive transgenic roots harbouring pJL-OFP-35S::GUS were further analysed by GUS staining solution (1 mM X-Gluc, 0.5 mM potassium ferrocyanide, 0.1% triton X-100, 100 mM sodium phosphate buffer pH 7.0) following a previously published protocol (Chen et al., 2003a). For the soybean plants containing OFP-positive transgenic hairy roots harbouring pJL-OFP or pJL-OFP-35S::GmSAMT1, the tap root, OFP-negative hairy roots and all but one transgenic hairy root were excised under the wounding site. The soybean plants with a single transgenic hairy root were subjected to SCN bioassays.

Analysis of SCN development in transgenic hairy roots

SCN bioassays using transgenic hairy roots were conducted following the protocol described by Melito et al. (2010). Transgenic hairy roots of twenty soybean plants harbouring identical constructs were loaded horizontally in a 13 × 9 × 2 cm sterilized inoculating tray containing one thin layer mixture of sterile sand and top soil (1:1), and the shoots were left out of inoculating tray. About 0.75 mL of inoculum, which contained about 5600 SCN eggs, was added to each root system. Roots were allowed to be inoculated with nematode eggs for infection for 7 days. Roots were then washed to remove extra SCN eggs and juvenile nematodes that had not penetrated the root tissue. Sterile vermiculite and cone-tainers was used to grow the infected chimeras in the growth chamber. Two weeks postinoculation, infected root samples were washed to remove vermiculite using tap water and cleared by 20% (v/v) bleach for no more than 4 min, and then stained by acid fuchsin according the procedure of Byrd et al. (1983). Nematodes at developmental stages, J2 (thin), J3 (sausage shaped), elongated male and J4 or adult female nematodes (fat lemon shaped) found within each infected root sample were tallied. The number of SCN was quite variable among individual plants for all the transgenic and control lines. The total number of SCN ranged from 0 to 105 per plant. Roots containing fewer than five nematodes were not used for further analysis. The ratio of the number of SCNs that developed beyond the J2 stage (J3+ J4+ adult) to the total number of nematodes per plant was used as an index to determine the resistance difference among the hairy roots harbouring their respective constructs. Then the ratios were normalized to the mean for control transgenic lines. Data were based on three independent biological replicate experiments when the TN02-275 background was used ($n > 10$ roots/independent lines). Data were based on two independent biological replicate experiments when 'Williams 82' background ($n > 15$ roots/independent lines) was used. The photographs of SCN were taken to show the representative stage using a ZEISS STEMI 2000-C microscope equipped with a Canon camera.

Quantitative reverse transcription PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed as previously reported (Mazarei *et al.*, 2011) to determine gene expression of *GmSAMT1* and four defence-related genes *GmICS1*, *GmICS2*, *GmNPR1-1* and *GmNPR1-2* in transgenic hairy roots. The soybean ubiquitin 3 gene (*GmUBI-3*, GenBank accession D28123) was used as a reference gene. The sequences of gene specific primers were as follows: *GmSAMT1* (forward) 5'-GTGAAATTGGAAGTCTTAAAGAAGGA-3', *GmSAMT1* (reverse) 5'-CAGATTCAAAGTCTAGAGCATTCCAC-3', *GmICS1* (forward) 5'-GGCCATTCGGAGCTGG-3', *GmICS1* (reverse) 5'-AGGAGAAGTGTGGTCTGTGAGAGA-3', *GmICS2* (forward) 5'-CATGGCCAT-TTCGGAGCTTA-3', *GmICS2* (reverse) 5'-CAGAAGATGGTCTGCGAATGG-3', *GmNPR1-1* (forward) 5'-AATTGACCAAGAGCTTCCGC-3', *GmNPR1-1* (reverse) 5'-CTACAGAAGCATCATTTTCAACATCTT-3', *GmNPR1-2* (forward) 5'-CAATTGACCAAGAGCTTCCAA-3', *GmNPR1-2* (reverse) 5'-CTATAGAAGCATCGTTTTCAACATCTC-3', *GmUBI-3* (forward) 5'-GTGTAATGTTGGATGTGTTCCC-3', and *GmUBI-3* (reverse) 5'-ACACAATTGAGTTCACACAAACCG-3'. Two types of transgenic hairy roots in the 'Williams 82' background, *GmSAMT1* overexpressing hairy roots and vector control hairy roots were used to compare the gene expression of *GmICSs* and *GmNPR1s* with/without SCN treatment, respectively. After the soybeans were inoculated with SCN eggs (SCN treatment) or sterile distilled water (control) for 1 week, the root tissues pooled from five soybeans containing the same construct were collected. Total RNA was isolated from three biological replicates of the respective hairy root tissues using RNeasy columns (Qiagen) following the manufacturer's instructions for plants. RNA was treated with RNase-Free DNase set (Qiagen) to remove genomic DNA, and total RNA (about 1.5 µg) was reverse-transcribed to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for qRT-PCR according to manufacturer's instructions. First-strand cDNA was diluted and placed in each qRT-PCR reaction. DNA accumulation was measured using SYBR Green as the reference dye. Genomic DNA contamination was monitored by PCR using RNA sample as template. Only one product was present in each reaction as indicated by the reference dye's dissociation curve of amplified products. The following PCR conditions were used: 50 °C for 2 min; 95 °C for 10 min; followed by 40 cycles of 95 °C for 15 s; 60 °C for 1 min; 72 °C for 30 s. All qRT-PCR assays were conducted in triplicate. PCR efficiencies for target and reference genes were equal between the target and control samples. Ct values and relative abundance were calculated using software supplied with the Applied Biosystems 7900 HT Fast Real-Time PCR system.

Statistical analysis

Resistance index (normalized proportion of nematodes developed beyond J2 stage over total nematodes) data among different types of transgenic soybean hairy roots were analysed using a one-way ANOVA using the mixed model of SAS (SAS 9.2 version). Least squares means of the resistance index were separated using Fisher's least significant difference (LSD) test. A $P < 0.01$ was considered to indicate statistical significance. Transcript abundance data from qRT-PCR of *GmSAMT1*, *GmICSs* and *GmNPR1s* genes were also analysed using a one-way ANOVA using the mixed model of SAS (Yuan *et al.*, 2008). Least squares means of relative expression were separated using Fisher's LSD test. A $P < 0.05$ was considered to indicate statistical significance.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. Demonstration of coexpression of two reporter genes in transgenic hairy roots of soybean. (a) Schematic representation of the construct used for coexpression of an orange fluorescent protein (OFP) reporter gene and a GUS reporter gene. '35S-Pro' and 'NOS-ter' represent the CaMV 35S promoter and the NOS terminator, respectively. (b) Analysis of transgenic hairy roots with the construct described in (a). OFP-positive transgenic hairy root (labelled with an asterisk) were separated from OFP-negative hairy root (labelled with a triangle) under OFP filter. (c) OFP-positive hairy roots were further demonstrated to be positive for GUS staining.