An (E,E)-α-farnesene synthase gene of soybean has a role in defence against nematodes and is involved in synthesizing insect-induced volatiles

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Keywords: sesquiterpene synthase, volatile, transgenic hairy roots.

Summary

Plant terpene synthase genes (TPSs) have roles in diverse biological processes. Here, we report the functional characterization of one member of the soybean TPS gene family, which was designated GmAFS. Recombinant GmAFS produced in Escherichia coli catalysed the formation of a sesquiterpene (E,E)-α-farnesene. GmAFS is closely related to (E,E)-α-farnesene synthase gene from apple, both phylogenetically and structurally. GmAFS was further investigated for its biological role in defence against nematodes and insects. Soybean cyst nematode (SCN) is the most important pathogen of soybean. The expression of GmAFS in a SCN-resistant soybean was significantly induced by SCN infection compared with the control, whereas its expression in a SCN-susceptible soybean was not changed by SCN infection. Transgenic hairy roots overexpressing GmAFS under the control of the CaMV 35S promoter were generated in an SCN-susceptible soybean line. The transgenic lines showed significantly higher resistance to SCN, which indicates that GmAFS contributes to the resistance of soybean to SCN. In soybean leaves, the expression of GmAFS was found to be induced by Tetranychus urticae (two-spotted spider mites). Exogenous application of methyl jasmonate to soybean plants also induced the expression of GmAFS in leaves. Using headspace collection combined with gas chromatography–mass spectrometry analysis, soybean plants that were infested with T. urticae were shown to emit a mixture of volatiles with (E,E)-α-farnesene as one of the most abundant constituents. In summary, this study showed that GmAFS has defence roles in both below-ground and above-ground organs of soybean against nematodes and insects, respectively.

Introduction

Soybean is a crop of global importance. Its yield can be significantly reduced due to diseases caused by microbial pathogens and infestation by herbivorous insects (Hartman et al., 2011). The approaches to managing biotic agents of soybean plants, similar to other major crops, include sound cultural practices, application of synthetic pesticides and deployment of resistant cultivars (Oerke, 2006). The development of disease/insect-resistant soybean may be assisted by the mechanistic elucidation of plant natural defences, especially the isolation of defence genes. The production of secondary metabolites is one strategy of plant natural defences against pathogens and insects (Bennett and Wallsgrove, 1994; Zhao et al., 2013). The most structurally diverse group of plant secondary metabolites is terpenoids, which have diverse roles in the interactions of plants with the environment, including serving as defences against pathogens and insects (Gershenson and Dudareva, 2007). The soybean genome has been fully sequenced (Schmutz et al., 2010). This valuable resource is expected to facilitate the identification of candidate genes involved in the biosynthesis of terpenoids, especially of those that have roles in natural defences of soybean plants. Terpene synthases (TPSs) are key enzymes for terpene biosynthesis. They catalyse the formation of terpenes from isoprenyl diphosphate substrates of various chain lengths (Degenhardt et al., 2009). In flowering plants, TPSs form a mid-sized gene family in each species (Chen et al., 2011). Over the past 10 years, we have been engaged in functional characterization of the TPS gene family in natural defences in several crop plants, including rice (Yuan et al., 2008), sorghum (Zhuang et al., 2012) and poplar trees (Danner et al., 2011). We have also embarked on a project to study the TPS family of soybean. A recent study (Liu et al., 2014) showed that the soybean TPS family (GmTPS) consists of more than 20 members. The expression of 21 GmTPS genes was examined in different soybean tissues. While many genes were found to be expressed in primarily reproductive organs, twelve GmTPS genes also showed different expression patterns in response to mechanical wounding (Liu et al., 2014). GmTPS was
determined to encode geraniol synthase, and transgenic tobacco overexpressing GmTPS3 showed increased resistance to cotton leaf worms (Liu et al., 2014).

In this study, we report the functional characterization of GmTPS21, which we designated GmAFS (G. max α-farnesene synthase). GmAFS was selected for this investigation because it was identified as one of the candidate defence genes against soybean cyst nematodes (SCNs) in our previous study (Mazarei et al., 2011). SCN is the most important pathogen of the soybean crop (Koenning and Wrather, 2010). Thus, it has been highly desired to identify and isolate soybean defence genes for genetic improvement of soybean for enhanced resistance against SCN. There were three objectives in this study. The first objective was to determine the biochemical function of the protein encoded by GmAFS. Terpene synthases can be categorized into monoterpene synthases, sesquiterpene synthases, and diterpene synthases, depending on the products they form (Chen et al., 2011). We used in vitro biochemistry to determine the specific biochemical activity of GmAFS. The second objective was to determine whether GmAFS indeed has a role in SCN resistance. For this objective, transgenic hairy roots overexpressing GmAFS were produced and assayed for SCN resistance. The third objective was to examine whether GmAFS has roles in soybean defence against other pests. In many plant systems, insect herbivory can induce the biosynthesis and emission of volatile terpenoids (Shrivastava et al., 2010). For the third objective, we specifically examined whether GmAFS has a role in soybean defence against insects in above-ground tissues.

Results
Expression of GmAFS is induced by SCN infection in SCN-resistant soybean

In our previous GeneChip analysis, the expression of GmAFS corresponding to Glyma.625.1.S1.at was shown to be significantly induced by SCN infection in the SCN-resistant soybean TN02-226, whereas gene expression was unchanged in SCN-infected susceptible (TN02-275) plants (Mazarei et al., 2011; see Supplemental Table 2 therein). As it is possible that false-positive results could occur in microarray experiments from cross-hybridization (Dai et al., 2002), quantitative reverse-transcription PCR (qRT-PCR) experiments were performed. First, root tissues were collected from the SCN-resistant soybean line TN02-226 and the SCN-susceptible soybean line TN02-275 with (3 days post-SCN inoculation) and without SCN infection. These samples were subject to qRT-PCR analysis for GmAFS. No significant difference was observed in the expression of GmAFS in the SCN-susceptible soybean with or without SCN infection. In contrast, the expression of GmAFS in the SCN-resistant soybean was significantly increased (about 2.5-fold) by SCN infection in comparison with that of the control roots without SCN infection (Figure 1).

Evolutionary relatedness of GmAFS with other terpene synthases

With the confirmation of GmAFS expression in relation to SCN infestation (Figure 1), our next objective was to determine the biochemical function of the protein encoded by GmAFS. To this end, we first performed phylogenetic analysis to determine the evolutionary relatedness of GmAFS with other TPSs including some with known functions. From the latest version of the annotated soybean genome, 22 putative full-length TPS genes including GmAFS (Glyma.13G321100) were identified. Phylogenetic trees were reconstructed using all TPSs from four sequenced dicot plants: soybean, Arabidopsis, apple and poplar. From this analysis, the soybean TPSs were determined to belong to five subfamilies: a, b, c, e/g and g (Figure 2). GmAFS is a member of the TPS-b subfamily. Most members of the TPS-b subfamily encode monoterpene synthase (Chen et al., 2011), except apple (E,E)-α-farnesene synthase (MdAFS) (Green et al., 2007) and poplar (E,E)-α-farnesene synthase (PtTPS2) (Danner et al., 2011), which are sesquiterpene synthases. GmAFS clustered together with MdAFS and PtTPS2 (Figure 2) and showed 53% and 51% sequence identity to MdAFS and PtTPS2, respectively. Besides sharing high overall sequence similarity, GmAFS, MdAFS and PtTPS2 exhibit conserved structural features. These include the aspartate-rich DDxxD motif, N5D/DTE motif, and H-α1 loop (Figure 3). The H-α1 loop of apple MdAFS has been previously demonstrated to function in the binding of the metal ion K+ (Green et al., 2009). Evolutionary relatedness, sequence homology and conserved structural features suggested that GmAFS encodes (E,E)-α-farnesene synthase.

GmAFS encodes a sesquiterpene synthase producing (E,E)-α-farnesene

Under the standard assay conditions (Zhuang et al., 2012), GmAFS did not show activity with either geranyl diphosphate or farnesyl diphosphate. As it has been demonstrated that the (E,E)-α-farnesene synthases from apple and poplar need K+ in addition to magnesium for catalytic activity (Danner et al., 2011; Green et al., 2009), we also performed assays containing K+. In the presence of K+, GmAFS converted farnesyl diphosphate into (E,E)-α-farnesene (Figure 4a). However, GmAFS was not able to convert geranyl diphosphate into monoterpene under the same conditions (Figure 4b).
Structural feature of the GmAFS model and comparison with that of MdAFS

With the experimental confirmation that GmAFS encodes (E,E)-\(\alpha\)-farnesene synthase (Figure 4), next, we compared the three-dimensional structures and the active sites of GmAFS and MdAFS. Homology models of GmAFS and MdAFS were generated by use of the structure of tobacco 5-epi-aristolochene synthase (PDB ID: SEAT) as template (Facchini and Chappell, 1992). The two models superpose well with a RMSD deviation of only 1.5 Å (Figure 5a). The models for GmAFS and MdAFS obtained with the X-ray structure of (E)-bornyl

![Figure 2 Phylogenetic tree of terpene synthases (TPSs) from soybean (blue), apple (red), poplar (pink), Arabidopsis (green) and representative ones from gymnosperm. PpCPS/KS is a diterpene synthase from the moss Physcomitrella patens; it resembles ancestral plant terpene synthases. GmAFS is the terpene synthase gene from soybean which was investigated in this study. MdAFS (GenBank accession AAO22848.2) and PtTPS2 (GenBank accession AEI52902) are known (E,E)-\(\alpha\)-farnesene synthases from apple and poplar, respectively. PaTPS-far (GenBank accession AAS47697) and Pt5 (GenBank Accession AAO61226) are (E,E)-\(\alpha\)-farnesene synthase from Picea abies and Pinus taeda, respectively. TPS-a, b, c, d, e/g and g depict subfamilies. All known (E,E)-\(\alpha\)-farnesenes are highlighted in yellow.](image-url)
diphosphate synthase (1N20), a monoterpene synthase (Whittington et al., 2002), as the template are also similar. The active site residues for GmAFS and MdAFS were found to be conserved. These residues as well as structural motifs (e.g., Hα1 loop, DDxxD motif and H helix) in GmAFS and MdAFS were well aligned (Figure 5b). It is of interest to note that the Hα1 loops from the two models superpose well, even though the residue corresponding to P486 in MdAFS is Ala (A480) in GmAFS.

Overexpressing GmAFS in transgenic hairy roots led to enhanced resistance to soybean cyst nematode

The expression pattern of GmAFS (Figure 1) strongly suggested that GmAFS may have a role in soybean defence against SCN. To test this hypothesis, we chose to use transgenic hairy root system, which has been proved in our previous studies to be a reliable system for evaluating candidate SCN-resistant genes (Lin et al., 2013). A SCN-susceptible line of soybean (Williams 82) was used

Figure 3  Protein sequence alignment of GmAFS with (E,E)-α-farnesene synthases from apple (MdAFS) and poplar (PtTPS2). Three conserved motifs among GmAFS, MdAFS1 and PtTPS2 are boxed: the ‘DDxxD’ motif, the ‘NSD/DTE’ motif and the ‘H-α1 loop’.

Figure 4  GC–MS total ion chromatograms of reaction products from a terpene synthase assay of E. coli-expressed GmAFS. (a) The terpene synthase assay of E. coli-expressed recombinant GmAFS using farnesyl diphosphate (FPP) as substrate showing the sesquiterpene synthase activity. (b) The terpene synthase assay of E. coli expressed GmAFS using geranyl diphosphate (GPP) as substrate showing no monoterpene synthase activity.
to produce transgenic hairy roots overexpressing GmAFS under control of CaMV35S promoter. For fast screening, transgenic hairy roots were generated to coexpress GmAFS and an orange florescence protein (OFP) reporter gene (Figure 6a) in the same cassette. As a negative control, transgenic hairy roots were also produced with a binary vector containing only the ORF reporter gene under the control of CaMV35S promoter (Figure 6b). There was no significant difference on generating the hairy roots between the GmAFS-overexpressing line and the vector control line. The mean number of adult females and cysts for the control line was about 17.0, whereas the mean number of adult females and cysts for GmAFS-overexpressing transgenic hairy roots was about 10.6. Significantly fewer cysts were observed in transgenic soybean hairy root overexpressing GmAFS than that from control transgenic hairy roots. The female index of transgenic hairy roots overexpressing GmAFS (approximately 60) was significantly lower than that of the control (artificially set to 100) (Figure 6c), which means that the transgenic soybean showed 40% decrease in susceptibility to SCN.

The expression of GmAFS in soybean leaves can be induced by herbivory and methyl jasmonate

Some terpene synthase genes are known to be expressed in multiple tissues and can be induced by multiple stresses (Fälldt et al., 2003). To understand whether GmAFS has roles in other biological processes other than defence against SCN, we examined the expression of GmAFS in leaves especially under stress conditions using qRT-PCR. The expression of GmAFS in leaves infested with Tetranychus urticae (two-spotted spider mite) was found to be 12-fold higher than its expression in control soybean leaves without T. urticae infestation (Figure 7). The jasmonate signalling pathway is essential for regulating plant defence responses to insect herbivory (War et al., 2012). To understand whether this pathway is also associated in regulating the expression of GmAFS, soybean plants were treated with methyl jasmonate and leaves were collected for gene expression analysis. The expression of GmAFS in the methyl jasmonate-treated plants was 11-fold higher than its expression in the control plants (Figure 7).
synthase gene from Arabidopsis (AtTPS03) does not support this hypothesis. In addition to the phylogenetic evidence presented in this paper (Figure 2), the Arabidopsis (E,E)-α-farnesene synthase behaves differently at the biochemical level. While GmAFS, MdAFS and PtTPS2 all use K+ and Mg2+ as cofactors, AtTPS03 uses only Mg2+ as a factor like most TPSs (Huang et al., 2010). It is certainly possible that the orthologous (E,E)-α-farnesene synthase gene was lost in Arabidopsis. The analysis of the putative orthologs of this gene in other species of Rosid will provide evidence for testing this hypothesis. Prior to this study, several (E,E)-α-farnesene synthase genes have been isolated from gymnosperms (Phillips et al., 2003), which belong to the TPS-d subfamily (Figure 2). Together, these results suggest that (E,E)-α-farnesene synthase genes have evolved multiple times in seed plants.

GmAFS has a role in soybean defence against SCN

The induced expression of GmAFS in SCN-resistant soybean suggested that GmAFS has a role in soybean defence against SCN (Figure 1). This hypothesis was supported with the overexpression of GmAFS in transgenic hairy roots of a soybean variety that is SCN susceptible (Figure 6). Interestingly, the expression pattern of GmAFS is highly similar to that of soybean salicylic acid methyltransferase gene (GmSAMT1) (Lin et al., 2013). The expression of GmSAMT1 in a SCN-susceptible line was not significantly changed with SCN infection. In contrast, its expression was significantly induced by SCN infection in a SCN-resistant line. Similarly, overexpression of GmSAMT1 in a SCN-susceptible line also led to enhanced resistance to SCN (Lin et al., 2013). The enhanced resistance of GmSAMT1-overexpressors to SCN was attributed to the changes in the salicylic acid signalling pathway (Lin et al., 2013). While the defence mechanism conferred by GmAFS is unclear, it is tempting to speculate that its product (E,E)-α-farnesene has nematicidal activity. A number of terpenoids have been demonstrated to be nematicidal (Ntalli et al., 2010; Oka et al., 2000). It will be interesting to determine whether the defence rendered by GmAFS is indeed due to the toxicity of (E,E)-α-farnesene or other mechanisms. It will also be interesting to determine whether GmAFS, GmSAMT1 and other SCN-resistant genes work concertedly to achieve SCN resistance.

GmAFS may be involved in indirect defence against insects

In addition to defence against SCN, GmAFS is suggested to have a role in soybean defence against herbivorous insects. When infested by soybean aphids, soybean plants emitted a mixture of volatile compounds including (E,E)-α-farnesene (Moraes et al., 2005). Aphid-induced volatiles from soybean plants were shown to attract soybean aphid’s natural enemies such as Syrphidae (Diptera), Chrysopidae (Neuroptera), and green lacewings (Mallinger et al., 2011). Volatile (E,E)-α-farnesene has been shown to be active signal in attracting natural enemies. Laboratory results showed that α-farnesene was attractive to parasitic wasps including Aphidius ervi, Coleomegilla maculata and Chrysoperla carnea (Du et al., 1998; Zhu et al., 1999). James (2005) also reported the parasitic mymarid wasp, Anagrus daanei, was attracted to farnesene in a field study. In the current study, T. urticae infestation induced the expression of GmAFS (Figure 7) and elevated emission of (E,E)-α-farnesene (Figure 8), suggesting that (E,E)-α-farnesene may have a role in attracting the natural enemies of T. urticae as well. In fact, (E,E)-α-farnesene is a common compound of herbivore-induced plant volatile blends from some plants such as bean, pear, apple and poplar (Boeve
et al., 1996; Danner et al., 2011; Du et al., 1998; Scutareanu et al., 2003). Therefore, (E,E)-α-farnesene might function in indirect defence pertaining to many insect pests for many plants, including soybean.

Other possible roles of GmAFS and its use for genetic improvement of soybean

In addition to its roles in defence against SCN and indirect defence against insects, GmAFS may also have other functions. For instance, the product of GmAFS may provide defence against bacterial pathogens, fungal pathogens and viruses. In a previous study, root-emitted sesquiterpene caryophyllene was shown to attract beneficial nematodes for defence against insects (Rasmann et al., 2005). It will be interesting to determine whether GmAFS-produced (E,E)-α-farnesene has a similar function. GmAFS (GmTPS21) was found to be highly expressed in stem and mature flowers and its expression increased nearly 25-fold after 2 h of wounding (Liu et al., 2014). Besides such functional studies, GmAFS presents a useful genetic improvement of soybean for enhanced defence against multiple biotic agents. We are in the process of producing transgenic soybean overexpressing GmAFS. Once produced, we will test these lines for defence function against individual pathogens and insects as well as the agronomic performance of the transgenic soybean in the field.

Experimental procedures

Plants, insects and plant treatments

Three soybean (Glycine max) lines were used in this study. These included ‘Williams 82’ and two genetically related breeding lines: TN02-226 and TN02-275, which are resistant and susceptible, respectively, to soybean cyst nematode (SCN) HG type 1.2.5.7 (race 2). TN02-226 and TN02-275 were used in our prior gene profiling to identify candidate SCN-resistant genes (Mazarei et al., 2011). Williams 82 soybean was used as the plant materials for SCN HG type 0 (race 3) bioassay and gene expression examination under two-spotted spider mites (Tetranychus urticae) and methyl jasmonate treatment. Chlorine gas-sterilized soybean seeds were germinated on autoclaved filter paper moistened with sterile distilled water. Three soybean seedlings were grown in a single pot under 150–200 μmol m−2 s−1 irradiance 12-h light/12-h dark cycle at 28 °C/25 °C for 21 days. For the methyl jasmonate treatment, 3-week-old Williams 82 seedlings were irrigated with 25 mL of 5 mM methyl jasmonate. After 24 h, leaves were harvested for gene expression analysis. For the insect treatment, 3-week-old Williams 82 soybean seedlings were transferred to the glasshouse for treatment with T. urticae. A total of 200 spider mites were used to infest one soybean plant. After 3 days of infestation, soybean plants were subjected to volatile profiling. After volatile profiling, leaves were harvested for gene expression analysis.

Database search and sequence analysis

TPS genes from soybean and apple were identified by analysing their respective genome sequences housed at Phytozome v9.1 (http://www.phytozome.net) using Blast search. The TPS genes from Arabidopsis and poplar were from a previous dataset (Chen et al., 2011). Multiple protein sequence alignment was performed using ClustalX 2.1 (Larkin et al., 2007). A maximum-likelihood tree was constructed using MEGA 6.0 (Tamura et al., 2013) with the Jones–Taylor–Thornton (JTT) model and bootstrapping of 1000 replicates.

Isolation of full-length cDNA of GmAFS

A full-length cDNA of GmAFS was isolated via RT-PCR from soybean roots infected by SCN. Total RNA was extracted from root tissues using the RNAeasy Plant Mini Kit (Qiagen, Valencia, CA), and DNA contamination was removed with DNase treatment following the manufacturers’ instructions. Then purified, total RNA was reverse-transcribed into first-strand cDNA in a 15 μL reaction volume using the First-Strand cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ) as previously described (Chen et al., 2003). The following primers were designed for cloning and semiquantitative RT-PCR as follows: GmAFS-F: 5′-ATGAATCAC TCATACGCAGAATCAAGT-3′ and GmAFS-R: 5′-CTATCTAAGGG GTTACAAACCAGTG-3′. The PCR program used to amplify the target genes was performed as follows: 94 °C for 2 min followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min 50 s, and a final extension at 72 °C for 10 min. PCR products were cloned into vector pEXPS/CT-TOPO and fully sequenced.
**Escherichia coli** expression of GmAFS and terpene synthase enzyme assay

The assays were conducted in standard assay conditions, as previously reported (Zhuang et al., 2012). To study the biochemical function of GmAFS, the above-mentioned protein expression vector pEXPS/CT-TOPO harbouring GmAFS was transformed into the *E. coli* strain BL21 (DE3) CodonPlus (Stratagene, La Jolla, CA). Fifty millilitres of liquid cultures of the bacteria harbouring the expression constructs were grown at 37 °C to an OD600 of 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) with the final concentration of 500 μM was added to the culture for induction, and the cells were kept cultured for 20 h at 18 °C. Then, the cells were collected by centrifugation and disrupted by a 4 × 30 sec sonication treatment in chilled extraction buffer (50 mM Mops, pH 7.0, with 5 mM MgCl₂, 5 mM sodium ascorbate, 0.5 mM PMSF, 5 mM dithiothreitol, and 10% (v/v) glycerol). The cell fragments were removed by centrifugation at 14 000 g, and the supernatant was desalted into assay buffer (10 mM Mops, pH 7.0, 1 mM dithiothreitol, 10% (v/v) glycerol) by passage through an Econopac 10DG column (Bio-Rad, Hercules, CA).

The enzyme assays for recombinant GmAFS were performed at 30 °C for 1 h, using 50 μL of the crude enzyme and 50 μL assay buffer with 10 μM substrate (geranyl diphosphate and farnesyl diphosphate, respectively), 10 mM MgCl₂, 0.05 mM MnCl₂, 50 mM KCl, 0.2 mM NaWO₄ and 0.1 mM NaF in a Teflon-sealed, screw-capped 1 mL GC glass vial. A solid-phase microextraction (SPME) fibre consisting of 100 μm polydimethylsiloxane (Supelco, Bellefonte, PA) was placed in the headspace of the vial for 10 min for collecting the volatile. For analysis of the absorbed reaction products, the SPME fibre was inserted directly into the injector of the gas chromatograph. GC-MS analysis and product identification were performed as described below.

**Homology models**

Homology models were built for both GmAFS and apple (E,E)-α-farnesene (MdAFS) based on the X-ray structure of Tobacco 5-epi-Aristolochene Synthase (PDB ID: SEAT) using the homology modelling program in the molecular operation environment (MOE). The composite plants were watered every other day and fertilized weekly with Peters Professional fertilizer (Scotts, Marysville, OH). The inoculation and bioassay for transgenic hairy root overexpressing GmAFS were performed as previously described (Lin et al., 2016). The inoculation was carried out in a growth chamber for 2 days with 16-h day length and 25 °C day/night temperature. The composite soybean plants with inoculated hairy roots were transplanted to sterile sand in 50-cm³ container (12 cm in length, 2.5 cm inside diameter) randomly arranged within the tray (Stuewe and Sons, Tangent, OR) and maintained for 35 days in the growth chamber with 16-h day length and 22 °C day/night temperature and 100–110 μmol/cm² s light intensity. The composite plants were watered every other day and fertilized with Peters Professional fertilizer (Scotts, Marysville, OH).

**SCN treatment for breeding lines and SCN bioassay on transgenic hairy root overexpressing GmAFS**

The cultures of SCN HG type 1.2.5.7 (race 2) and SCN HG type 0 (race 3) were maintained in Dr. Arelli’s laboratory (Arelli et al., 2000). SCN HG type 1.2.5.7 (race 2) was used in 6-day treatment on the soybean breeding lines, TN02-226 (SCN resistant) and TN02-275 (SCN susceptible) (Mazarei et al., 2011). SCN HG type 0 (race 3) was used for in 35-day bioassay on transgenic hairy root overexpressing GmAFS. Active second-stage juvenile (J2) nematodes (1000 J2 SCN/plant) were used for inoculation. SCN inoculation and bioassay for transgenic hairy root overexpressing GmAFS were performed as previously described (Lin et al., 2016). The inoculation was carried out in a growth chamber for 2 days with 16-h day length and 25 °C day/night temperature. The composite soybean plants with inoculated hairy roots were transplanted to sterile sand in 50-cm³ container (12 cm in length, 2.5 cm inside diameter) randomly arranged within the tray (Stuewe and Sons, Tangent, OR) and maintained for 35 days in the growth chamber with 16-h day length and 22 °C day/night temperature and 100–110 μmol/cm² s light intensity. The composite plants were watered every other day and fertilized with Peters Professional fertilizer (Scotts, Marysville, OH).
harbouring pJL-OPF-35S:Gm4FS at the cyst stage was calculated based on female index. Each of the SCN bioassays was conducted with four replicates.

**Plant volatile collection and identification**

Volatile emitted from the *T. urticae*-treated and control soybean plants were collected in an open headspace sampling system (Analytical Research Systems, Gainesville, FL). Three plants grown in a pot with root systems wrapped with aluminium foil were placed in a glass chamber (30 cm high × 10 cm diameter), with a removable O-ring snap lid with an air outlet port. The charcoal-purified air was passed into the chamber at a flow rate of 0.8 L min⁻¹ from the top through a Teflon® hose. Volatiles were collected by pumping air from the chamber through a SuperQ volatile collection trap (Analytical Research Systems, Gainesville, FL). After 16-h collection, 100 μL of methylene chloride containing 1-octanol (0.003%) as an internal standard was used to elute the volatiles into a glass tube for quantification. The volatile analysis was performed in triplicate to confirm the volatile products.

Plant volatiles and volatile terpenoids from TPS enzyme assays were analysed by a Shimadzu QP5050A quadrupole mass selective detector. Compounds’ separation was performed on a Restek SHRXLB column with 30 m × 0.25 mm internal diameter × 0.25 μm thickness (Shimadzu, Columbia, MD). Helium was used as the carrier gas at flow rate of 1.7 mL min⁻¹, and a splitless injection (injection temperature 250 °C) was used. A temperature gradient of 5 °C min⁻¹ from 60 °C (6 min hold) to 300 °C was applied. Products were identified based on the National Institute of Standards and Technology (NIST) mass spectral database by comparing of retention times and mass spectra with authentic reference compounds if available. Compound quantification was performed as previously reported (Chen et al., 2009). Representative single-ion peaks of each compound were integrated and compared with the equivalent response of the internal standard (single-ion method).

**Statistical analysis**

Statistical analysis for gene expression and female index was tested with a one-way ANOVA followed by Fisher’s LSD with an alpha level of 0.05 using R software (version 3.1.0) (R Foundation for Statistical Computing, Vienna, Austria).

**Acknowledgements**

This work was supported by the Tennessee Soybean Promotion Board. We thank Dr. Tarek Hewezi’s laboratory for sharing with us the SCN race 3. We would also like to thank Dana Pekarchick and Susan Thomas (USDA-ARS, Jackson, TN, USA) for assistance with maintaining the SCN cultures.

**Conflict of interest**

The authors declare that they have no competing interests.

**References**

Arelli, P.R., Sleper, D.A., Yue, P. and Wilcox, J.A. (2000) Soybean reaction to races 1 and 2 of *Heterodera glycines*. *Crop Sci*. 40, 824–826.

Bennett, R.N. and Wallisgrove, R.M. (1994) Secondary metabolites in plant defense mechanisms. *New Phytol*. 127, 617–633.

Boeve, J.L., Lengwiler, U., Dom, S., Turlings, T.C.J. and Tolsten, L. (1996) Volatiles emitted by apple fruitlets infected by larvae of the European apple sawfly. *Phytochem. Lett*. 42, 372–381.

Chen, H., Nelson, R.S. and Sherwood, J.L. (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. *Biotechniques*, 16, 664–670.

Chen, F., Tholl, D., D’Auria, J.C., Farooq, A., Pichersky, E. and Gershenson, J. (2003) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. *Plant Cell*, 15, 481–494.

Chen, F., Al-Kindawi, H., Joyce, B., Zhao, N., Kühler, T.G., Degenhardt, J. and Stewart, C.N. (2009) Within-plant distribution and emission of sesquiterpenes from *Coelopa officinalis*. *Plant Physiol. Biochem*. 47, 1017–1023.

Chen, F., Tholl, D., Bohlmann, J. and Pichersky, E. (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. *Plant J*. 66, 212–229.

Dai, H.Y., Meyer, M., Stepaniants, S., Ziman, M. and Stoughton, R. (2002) Use of hybridization kinetics for differentiating specific from non-specific binding to oligonucleotide microarrays. *Nucleic Acids Res*. 30, e86.

Danner, H., Boeckler, G.A., Irmisch, S., Yuan, J.S., Chen, F., Gershenzon, J., Unsicker, S.B. et al. (2011) Four terpene synthases produce major compounds of the gypsy moth feeding-induced volatile blend of *Populus trichocarpa*. *Phytochemistry*, 72, 897–908.

Degenhardt, J., Kübler, T. and Gershenson, J. (2009) Monoterpenes and sesquiterpenes synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry*, 70, 1621–1637.

Du, Y.J., Poppy, G.M., Powell, W., Pickett, J.A., Wadhams, L.J. and Woodcock, C.M. (1998) Identification of semiochemicals released during aphid feeding that attract parasitoid *Aphidius ervi*. *J. Chem. Ecol*. 24, 1355–1368.

Facchin, P.J. and Chappell, J. (1992) Gene family for an elicitor-induced sesquiterpene cyclase in tobacco. *Proc Natl Acad. Sci. USA*. 89, 11088–11092.

Fäldt, J., Martin, D., Miller, B., Rawat, S. and Bohlmann, J. (2003) Traumatic resin defense in Norway spruce (*Picea abies*: methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol. Biol*. 51, 119–133.

Gershenson, J. and Dudareva, N. (2007) The function of terpene natural products in the natural world. *Nat. Chem. Biol*. 3, 408–414.

Green, S., Friel, E.N., Matich, A., Beuning, L.L., Cooney, J.M., Rowan, D.D. and MacRae, E. (2007) Unusual features of a recombinant alpha-alpha-farnesene synthase. *Phytochemistry*, 68, 176–188.

Green, S., Squire, C.J., Neuwetzenhuisen, N.J., Baker, E.N. and Laing, W. (2009) Defining the potassium binding region in an alpha terpene synthase. *J. Biol. Chem*. 284, 8661–8669.

Hartman, G.L., West, E.D. and Herman, T.K. (2011) Crops that feed the World 2: Soybean-worldwide production, use, and constraints caused by pathogens and pests. *Food Sec*. 3, 5–17.

Huang, M.S., Abel, C., Sohrabi, R., Petri, J., Haupt, I., Cosimano, J., Gershenson, J. et al. (2010) Variation of herbivore-induced volatile terpenes among *Arabidopsis* ecotypes depends on allelic differences and subcellular targeting of two terpene synthases, TSF02 and TSF03. *Plant Physiol*. 153, 1293–1310.

James, D. (2005) Further field evaluation of synthetic herbivore-induced plant volatiles as attractants for beneficial insects. *J. Chem. Ecol*. 31, 481–495.

Koebrich, S.R. and Wrather, J.A. (2010) Suppression of soybean yield potential in the continental United States from plant diseases estimated from 2006 to 2009. *Plant Health Prog*.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F. et al. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics*, 23, 2947–2954.

Lin, J., Mazarei, M., Zhao, N., Zhu, J.I.W., Zhuang, X.F., Liu, W.S., Pantalone, V.R. et al. (2013) Overexpression of a soybean salicylic acid methyltransferase gene confers resistance to soybean cyst nematode. *Plant Biotechnol. J*. 11, 1135–1145.

Lin, J., Mazarei, M., Zhao, N., Hatcher, C.N., Wuddineh, W.A., Rudis, M., Tschaplinski, T.J. et al. (2016) Transgenic soybean overexpressing GmSAMT1 exhibits resistance to multiple-HG types of soybean cyst nematode *Heteroderma glycines*. *Plant Biotechnol. J*. 14, 2100–2109.
Mallinger, R.E., Hogg, D.B. and Gratton, C. (2011) Methyl salicylate attracts methyl jasmonate to resistant and susceptible soybean lines infected with soybean cyst nematode. *Theor. Appl. Genet.* 123, 1193–1206.

Moraes, M.C.B., Laumann, R., Sujii, E.R., Pires, C. and Borges, M. (2005) Induced volatiles in soybean and pigeon pea plants artificially infested with the neotropical brown stink bug, *Euschistus heros*, and their effect on the egg parasitoid, *Teuknomus podisi*. *Entomol. Exp. Appl.* 115, 227–237.

Niblack, T.L. (2005) Soybean cyst nematode management reconsidered. *Plant Dis.* 89, 1020–1026.

Oka, Y., Nacar, S., Putievsky, E., Ravid, U., Yaniv, Z. and Spiegel, Y. (2000) Nematicidal activity of essential oils and their components against the root-knot nematode. *Phytopathology*, 90, 710–715.

Phillips, M.A., Wildung, M.R., Williams, D.C., Hyatt, D.C. and Croteau, R. (2003) cDNA isolation, functional expression, and characterization of (−)-α-pinene synthase and (−)-α-pinene synthase from loblolly pine (*Pinus taeda*): Stereocontrol in pinene biosynthesis. *Arch. Biochem. Biophys.* 411, 267–276.

Rasmann, S., Köllner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., Gershenzon, J. et al. (2005) Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature*, 434, 732–737.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L. et al. (2010) Genome sequence of the palaeopolyploid soybean. *Nature*, 463, 178–183.

Scutareanu, P., Bruin, J., Posthumus, M.A. and Drukker, B. (2003) Constitutive and herbivore-induced volatiles in pear, alder and hawthorn trees. *Chemoecology*, 13, 63–74.

Shrivastava, G., Rogers, M., Wszelaki, A., Panthee, D.R. and Chen, F. (2010) Plant volatiles-based insect pest management in organic farming. *Crit. Rev. Plant Sci.* 29, 123–133.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729.

War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., Ignacimuthu, S. and Sharma, H.C. (2012) Mechanisms of plant defense against insect herbivores. *Plant Signal Behav.* 7, 1306–1320.

Whittington, D.A., Wise, M.L., Urbansky, M., Coates, R.M., Croteau, R.B. and Christianson, D.W. (2002) Bornyl diphenolate synthase: structure and strategy for carbocation manipulation by a terpenoid cyclase. *Proc. Natl Acad. Sci. USA*, 99, 15375–15380.

Yuan, J.S., Reed, A., Chen, F. and Stewart, C.N. (2006) Statistical analysis of real-time PCR data. *BMC Bioinformatics*, 7, 85.

Yuan, J.S., Köllner, T.G., Wiggins, G., Grant, J., Degenhardt, J. and Chen, F. (2008) Molecular and genomic basis of volatile-mediated indirect defense against insects in rice. *Plant J.* 55, 491–503.

Zhao, N., Wang, G.D., Norris, A., Chen, X.L. and Chen, F. (2013) Studying plant secondary metabolism in the age of genomics. *Crit. Rev. Plant Sci.* 32, 369–382.