Expression of lima bean terpene synthases in rice enhances recruitment of a beneficial enemy of a major rice pest

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ABSTRACT

Volatile terpenoids play a key role in plant defence against herbivory by attracting parasitic wasps. We identified seven terpene synthase genes from lima bean, Phaseolus lunatus L., following treatment with either the elicitor alamethicin or spider mites, Tetranychus cinnabarinus. Four of the genes (Pltps2, Pltps3, Pltps4 and Pltps5) were up-regulated with their derived proteins phylogenetically clustered in the TPS-g subfamily and PlTPS3 positioned at the base of this cluster. Recombinant PlTPS3 was able to convert geranyl diphosphate and farnesyl diphosphate to linalool and (E)-nerolidol, the latter being precursor of the homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT). Recombinant PlTPS4 showed a different substrate specificity and produced linalool and (E)-nerolidol, as well as (E,E)-geranyllinalool from geranylgeranyl diphosphate. Transgenic rice expressing Pltps3 emitted significantly more (S)-linalool and DMNT than wild-type plants, whereas transgenic rice expressing Pltps4 produced (S)-linalool, DMNT and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). In laboratory bioassays, female Cotesia chilonis, the natural enemy of the predatory mite Phytoseiulus persimilis (Dicke et al. 1990). It was shown to be released by lima bean P. lunatus after it was damaged and involved in the recruitment of beneficial enemies (Turlings et al. 1990). It was first identified in maize Zea mays L. as a floral volatile that attracted females of the parasitic wasp Cotesia marginiventris Cresson, a natural enemy of beet armyworm Spodoptera exigua (Turlings and Tumlinson 1992). A mixture of terpenoid and shikimate-pathway-derived volatiles was shown to be released by lima bean P. lunatus after it was attacked by the spider mite Tetranychus urticae, which attracts the predatory mite Phytoseiulus persimilis (Dicke et al. 1990). It was reported that plants in the genus Desmodium, a forage legume intercropped with cereal crops, repel stemborer moths and attract their natural enemies (Khan et al. 2014;
The generation of genetically modified plants with overexpressed TPS genes is useful in studying the physiological and ecological functions of specific terpenoids (Aharoni et al. 2005; Degenhardt et al. 2009; Brillard et al. 2013; Gao et al. 2015; Zhang et al. 2015). For example, targeting of the TPS gene FaNES1 of strawberry Fragaria × ananassa, into A. thaliana mitochondria resulted in the recruitment of carnivorous predatory mites through the enhanced production of (E)-nerolidol and DMNT (Kappers et al. 2005). Overexpression of P. lunatus TPS gene Ptps2 in Lotus japonicus enhanced the capability of transgenic plants in attracting predatory mites (Brillard et al. 2013), whilst overexpression of wheat FPP synthase genes (FPS) in A. thaliana resulted in repellency of the peach-potato aphid, Myzus persicae (Zhang et al. 2015). The transgenic approach was also used to restore maize resistance to entomopathogenic nematodes (EPNs) by transforming non-emitting maize lines with a (E)-caryophyllene synthase gene (TPS23) for the production of (E)-caryophyllene in maize roots emitting maize lines with a (E)-caryophyllene synthase gene (Brillada et al. 2013), whilst overexpression of wheat FPP synthase genes in A. thaliana resulted in repellency of the peach-potato aphid, Myzus persicae (Zhang et al. 2015). The transgenic approach was also used to restore maize resistance to entomopathogenic nematodes (EPNs) by transforming non-emitting maize lines with a (E)-caryophyllene synthase gene (TPS23) for the production of (E)-caryophyllene in maize roots emitting maize lines with a (E)-caryophyllene synthase gene (Brillada et al. 2013), whilst overexpression of wheat FPP synthase genes (FPS) in A. thaliana resulted in repellency of the peach-potato aphid, Myzus persicae (Zhang et al. 2015). The transgenic approach was also used to restore maize resistance to entomopathogenic nematodes (EPNs) by transforming non-emitting maize lines with a (E)-caryophyllene synthase gene (TPS23) for the production of (E)-caryophyllene in maize roots emitting maize lines with a (E)-caryophyllene synthase gene (Brillada et al. 2013), whilst overexpression of wheat FPP synthase genes (FPS) in A. thaliana resulted in repellency of the peach-potato aphid, Myzus persicae (Zhang et al. 2015). The transgenic approach was also used to restore maize resistance to entomopathogenic nematodes (EPNs) by transforming non-emitting maize lines with a (E)-caryophyllene synthase gene (TPS23) for the production of (E)-caryophyllene in maize roots emitting maize lines with a (E)-caryophyllene synthase gene (Brillada et al. 2013), whilst overexpression of wheat FPP synthase genes (FPS) in A. thaliana resulted in repellency of the peach-potato aphid, Myzus persicae (Zhang et al. 2015).

Previously, P. lunatus was found to emit volatile terpenoids following exposure to alamethicin (ALA), a peptide elicitor from the fungus Trichoderma viride (Engelberth et al. 2001), and upon exposure to the feeding of the spider mite Tetranychus urticae (Arimura et al., 2000a). Here, we report on isolation of TPS genes from P. lunatus following either ALA or T. cinnabarinus treatment, confirmation of the products of their coding syntheses in vitro and expression of these genes in rice plants to validate terpenoid production in vivo. We also use the transgenic plants in bioassays with parasitic wasps, C. chilonis to demonstrate their ecological potential, that the transformed plants can produce an inducible volatile signal known to attract parasitoids of rice plants against herbivory.

MATERIALS AND METHODS

Plants, spider mites and parasitic wasps

Lima bean plants, P. lunatus L. cv. Sieva, were grown in a growth chamber (25 ± 2 °C, 50%–70% RH, 16:8 L:D regime). Wild-type (WT) and transgenic rice plants, Oryza sativa L. ssp. japonica, variety Zhonghua 11 (ZH11) were grown in plastic pots in a greenhouse at 25 °C with the same photoperiod. Carmine spider mites, T. cinnabarinus, a pest of P. lunatus, were obtained from the lab of Dr. Xuenong Xu, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, and reared on P. vulgaris L., in a growth chamber (20–30 °C, 60–70% RH, 16:8 L:D regime). Parasitic wasps, C. chilonis, a natural enemy of the striped rice stemborer, C. suppressalis Walker (Lepidoptera: Crambidae), were reared on C. suppressalis larvae, maintained on an artificial diet in a climate-controlled room (25 °C, c 16:8 L:D). Female and male wasps were separated and distinguished by the length of antennae, with female antennae being much shorter than male antennae (personal observation).

Alamethicin and spider mite treatment of P. lunatus

Two-week-old P. lunatus plants were either treated with alamethicin (ALA, 10 μg mL$^{-1}$, Toronto Research Chemicals, Canada), 100 female T. cinnabarinus or left untreated as mock-treated control. ALA was diluted in tap water (10 mL) and then applied to the petioles of P. lunatus. All treatments were carried out in a growth chamber (25 ± 2 °C, 50–70% RH, 16:8 L:D) as previously described (Arimura et al. 2000b; Engelberth et al. 2001). Twenty four hours after treatment, plants were used in volatile collections or RNA extractions.

Volatile collection and analysis

Volatile sampling from ALA-treated, T. cinnabarinus-treated and mock-treated P. lunatus was undertaken using solid-phase microextraction (SPME) at 24 h after the treatments. Briefly, leaf material was inserted into a glass vial with a septum in the lid. A 50/30-μm divinylbenzene/carboxen/polydimethylsiloxane SPME fibre (Supelco; Sigma-Aldrich, http://www.sigmaaldrich.com) was inserted through the septum and exposed for 60 min at 40 °C. Volatile collection from 45 to 50-day-old transgenic rice plants was undertaken by air entrainment. Transgenic plants were enclosed in a sealed glass chamber, and air (purified using activated charcoal) containing the volatiles was drawn at a rate of 750 mL min$^{-1}$ for 24 h through glass tubes containing Tenax (50 mg, 60/80 mesh; Supelco, Bellefonte, PA, USA). The trapped volatiles were eluted with hexane (0.4 mL). Solid-phase microextraction and air entrainment samples were analysed by GC–MS (4D GC × GC-TOF-MS) (LECO, San Jose, USA). For SPME samples, volatiles adsorbed onto the SPME fibre were removed by thermal desorption. For air entrainment samples, aliquots of samples (1 μL) were analysed. All analyses were carried out using a DB5-MS column (30 m × 0.25 mm ID × 0.25 μm film thickness; Agilent Technologies) with a split/splitless injector (splitless mode, 220 °C). Helium was used as the carrier gas at 1 mL min$^{-1}$; the oven temperature was programmed to raise from 50 to 60 °C (5 min hold) with a rate of 5 °C min$^{-1}$ and then raised to 250 °C with a rate of 10 °C min$^{-1}$ (5 min hold). The transfer line temperature was 250 °C; ion source temperature was 250 °C. Ionization was by electron impact (70 eV), and the scan range was between m/z 50 and 650. Volatiles were identified by comparison of their GC retention times and mass spectra with authentic reference.
compounds, and for entrainment samples, identities were also confirmed by co-injection of samples with authentic standards. DMNT and TMTT were synthesized from geraniol and (E,E)-farnesol, respectively (Leopold 1990). (S)-Linalool, (E)-ocimene and (E)-caryophyllene were purchased from Sigma-Aldrich (St. Louis, MO). Linear calibrations were obtained from 0.5 to 50 ng μL⁻¹ for linalool (R² = 0.9991), DMNT (R² = 0.9958) and TMTT (R² = 0.9910). Differences in volatile content between samples were analysed with variation (ANOVA). For entantioselective GC analysis of (S)-linalool, an Astec® CHIRALDEX® B-DM capillary GC column (50 m × 0.25 mm, df 0.12 μm, Supelco, Sigma-Aldrich, Bellefonte, USA) was used. We also quantified the volatile components emitted by the transgenic plants and made synthetic blends that mimic natural blends (Fig. S1) to confirm the attractiveness of the volatiles from the transgenic rice plants to parasitic wasps.

De novo transcriptome sequencing and digital gene expression analysis

TRIZol reagent (Invitrogen) was used to isolate total RNAs from ALA-treated, T. cinnabarinus-treated and mock-treated P. lunatus plant samples. Four cDNA libraries (ALA-treated and ALA-mock treated, T. cinnabarinus-treated, T. cinnabarinus-mock treated) were constructed as previously described (Wang et al. 2010) and sequenced separately on an Illumina sequencing GAII platform as previously reported (Yu et al. 2014). All reads of four libraries were pooled together and assembled to a single de novo transcriptome dataset using Trinity (v. 20140717) (Grabherr et al. 2011). The assembly was BLASTx searched against the NCBI non-redundant (nr) (http://www.ncbi.nlm.nih.gov/) and UniProt protein databases (http://www.uniprot.org/). The transcriptome reads were deposited in the NCBI Sequence Read Archive, and the accession number is SRX894594 (Li et al. 2015). For global analysis of volatile metabolism, 12 samples from ALA-treated and ALA-mock treated, T. cinnabarinus-treated and T. cinnabarinus-mock treated (three replicates per treatment) were used to establish digital gene expression (DGE) libraries (NCBI SRA number is SRP099506). Clean reads of each sample were aligned with the lima bean transcriptome assembly using Bowtie v2.0.6 (Langmead and Salzberg 2012). For quantification of gene expression, the read numbers mapped to each gene were counted using HTSeq v0.5.4p3, and the reads per kb per million reads (RPKM) was calculated (Mortazavi et al. 2008). Differential expression analysis was performed using the DESeqSeq R package v1.12.0 (Anders and Huber 2010). To minimize false positive results, the P values were adjusted, and Q-values were used for significance tests. The Q-values of 0.005 and log2 (fold change) of 1 were served as the thresholds of significance for differential expression. Putative TPS genes from P. lunatus (Pltps) were identified according to the methods described by Li et al. (2012). Briefly, the hidden Markov Models (hmm) search command in the HMMER package (Wistrand and Sonnhammer 2005) was used to search for the P. lunatus transcriptome assembly dataset with pfam PF01397 and PF03936 of TPS domains with a threshold of 1e 2. Phylogeny trees were produced using neighbour-joining method with MEGA6.06 (Tamura et al. 2013).

Production of P. lunatus recombinant proteins in E. coli

The full-length open reading frame (ORF) of the P. lunatus TPS genes Pltps3 and Pltps4 was cloned by rapid amplification of cDNA ends (RACE) PCR. The primers for RACE are listed in Table S1. The full-length ORF of Pltps3 and Pltps4 were cloned from P. lunatus cDNA using primers designed with XhoI/BamH I and BamH I/EcoRI restriction enzymes sites at the 5'/3' ends of the primers, respectively (Table S1). The PCR-amplified product was digested and cloned into the corresponding sites of pET30a vector (Novagen, Madison, WI) to generate expression constructs pET30a-PlTPS3 and pET30a-PlTPS4. The final construct was transformed with E. coli BL21 (DE3) competent cell (TansGenBiotech, Beijing). Recombinant E. coli cells were grown in 2L LB medium with kanamycin at 100 mg mL⁻¹. The cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1 mM and further grown for overnight at 18 °C. The cells were centrifuged and re-suspended in the buffer containing 50 mM MOPS, pH 7.0, 5 mM MgCl2, 5 mM sodium ascorbate, 0.5 mM PMSF, 5 mM dithiothreitol and 10% (v/v) glycerol. The re-suspended cells were disrupted by sonication. Cell extracts were centrifuged, and the expressed proteins in the supernatant were purified by passage through HisTrap affinity columns (GE Healthcare Biosciences, Uppsala, Sweden) into the assay buffer (10 mM MOPS, pH 7.0, 1 mM dithiothreitol, 10% (v/v) glycerol).

Functional characterization of P. lunatus recombinant proteins

Purified recombinant PITPS proteins were assayed in vitro for enzyme activity as follows: 200 μL purified protein (4 mM) was added into a glass vial containing 800 μL of the assay buffer containing 10 mM MgCl2, 0.05 mM MnCl2, 0.2 mM NaWO4 and 0.1 mM NaF, and supplemented with either 10 μM GPP, FPP or GGPP (Sigma-Aldrich). Volatiles from enzyme assays were collected and analysed by SPME as described above (volatile collection and analysis).

Generation of transgenic rice lines

The coding sequences of Pltps3 and Pltps4 were PCR amplified from P. lunatus cDNA and inserted into the pCAMBIA1300-EPSPS vector with a ubiquitin promoter and nos terminator (Yi et al. 2016). The overexpression constructs were transformed into O. sativa L. ssp. japonica, variety ZH11 using
Agrobacterium tumefaciens strain EHA105. The Agrobacterium-mediated transformation and regeneration of transgenic plants were conducted as previously described (Tang et al. 2006). After glyphosate selection, T1 plants were used for further analyses of gene expression, volatile emission and insect behavioural studies.

Quantification of terpene synthase gene expression in plants

The total RNAs of WT rice (O. sativa var. Zhonghua 11) plants and transgenic rice plants OSPT3 and OSPT4 expressing Pltps3 and Pltps4, respectively, were isolated with TRIzol reagent (Invitrogen). The total RNAs of the transgenic rice plants and the lima bean plants were reverse transcribed using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). The expression profiles of the Pltps3 and Pltps4 transcripts in lima bean P. lunatus plants, WT and the transgenic rice plants were evaluated by real-time quantitative PCR (qPCR).

The primers were designed using IDT Primer Quest (http://www.idtdna.com/primerquest/Home/Index) and listed in Table S1. Quantitative PCR was performed on the ABI Prism® 7500 (Applied Biosystems, Carlsbad, CA, USA) and the reactions conducted in 20 μL reaction mixture containing 10 μL 2 × GoTaq qPCR Master Mix (Promega, Madison, WI, USA), 10 μM primers, 1 μL cDNA (200 ng μL⁻¹) and 8 μL nuclease-free water. The following procedure was used for qPCR: 2 min at 95 °C, 40 cycles of 30 s at 95 °C and 1 min at 60 °C. Relative RNA variations were normalized and corrected with the level of the reference actin gene of lima bean plact1 (GenBank accession no. DQ159907).

Parasitoid behaviour assays

Y-Tube olfactometer assays were conducted to investigate the behavioural responses of the specialist parasitoid wasp C. chilonis, the natural enemy of the striped rice stemborer, C. suppressalis, to transgenic rice plants (OSPT3 and OSPT4) and their volatiles. Responses to different doses of synthetic volatiles were also tested. The olfactometer dimensions were 10 cm length for the stem and both arms with a 1.5 cm internal diameter. The air flow was maintained at 250 mL/min.

In each experiment, a single female wasp was released at the base of the Y-tube stem and its behaviour observed for 10 min. Wasps not making any choice during that time were recorded as non-responders. Wasps entering into more than the half length of the olfactometer arms and lingering for at least 10 s were recorded as responders. After 10 individuals were tested, the two arms were interchanged. The Y-shaped tube was cleaned with acetone followed by ethanol and then air-dried after each treatment. The olfactory experiments were performed between 10:00 h and 16:00 h. The following comparative experiments were carried out to examine wasp responses: (1) transgenic rice line OSPT3 (O. sativa expressing Pltps3) versus WT (ZH11); (2) transgenic rice line OSPT4 (O. sativa expressing Pltps4) versus WT (ZH11); (3) volatiles collected from OSPT3 versus volatiles from WT (ZH11); (4) volatiles collected from OSPT4 versus volatiles collected from WT (ZH11); (5) synthetic blend of volatiles from OSPT3 versus volatiles from WT (ZH11); (6) synthetic blend of volatiles from OSPT4 versus volatiles from WT (ZH11); (7) (S)-linalool (100, 10 and 1 ng in mineral oil) versus mineral oil; (8) DMNT (1 ng in mineral oil) versus mineral oil; and (9) TMTT (1 ng in mineral oil) versus mineral oil. The behavioural responses of the female wasps were assessed using Chi-square ($\chi^2$) analysis.

RESULTS

Candidate terpene synthase genes from P. lunatus involved in terpenoid production

GC–MS analysis of volatiles collected from P. lunatus plants following ALA treatment detected the presence of DMNT and TMTT, and, following spider mite T. cinnibarinus treatment, the presence of (E)-ocimene, linalool, DMNT, TMTT and (E)-caryophyllene (Fig. S1). The emission of DMNT and TMTT from ALA-treated and spider mite-treated P. lunatus plants was significantly higher than that from mock-treated plants (P < 0.05, Student’s t-test) (Fig. S2). Screening of the transcriptomes of the ALA-treated and spider mite-treated P. lunatus plants by homologous BLAST searching with an E-value threshold of 1e 2 and DGE analysis identified seven TPS gene homologues named as Pltps1–Pltps7 (Table S2). The sequences of newly identified TPS genes were deposited to the NCBI database with the accession numbers of KY574604 for Pltps1, KY574605 for Pltps3, KY574606 for Pltps4, KY379970 for Pltps5, KY574607 for Pltps6 and KY574608 for Pltps7. A neighbour-joining phylogenetic tree was constructed to compare the sequence similarity relationships of the derived protein sequences (PITPS1–PITPS7) to other angiosperm TPSs (Fig. 1). Of the seven PITPSs, three TPSs (PITPS1, PITPS5 and PITPS7) belonged to the TPS-b subfamily. PITPS5 was very close to a (E)-caryophyllene synthase (NCBI ID: AEP17005.1) of the common grape vine Vitis vinifera, whilst PITPS1 and PITPS7 were very close to the isoprene synthase of V. vinifera and the black locust, Robinia pseudoacacia, respectively (Fig. 1). The other four PITPSs (PITPS2, PITPS3, PITPS4 and PITPS6) were clustered into the TPS-g subfamily. PITPS2 was close to the 3-nerolidol synthase gene of soybean (NCBI ID: XP_003528418) and to (E)-ocimene synthase MtTPS3 from M. truncatula (NCBI ID: EU194553) (Arimura et al. 2008). PITPS2 was previously reported to be linalool/(E)-nerolidol/(E,E)-geranylinalool synthase and involved in TMTT biosynthesis (Brillada et al. 2013). PITPS3, PITPS4 and PITPS6 have not been previously characterized. PITPS3 was not clustered with any other annotated TPSs and was positioned at the base of this cluster as an ancient member of TPS-g subfamily (Fig. 1). PITPS4 was also clustered in this subfamily, suggesting a
close relationship to the previously characterized PITPS2. PITPS6 was clustered with linalool/(E)-nerolidol synthase (LIN/NES) group in the TPS-g subfamily. Digital gene expression analysis of the four identified synthase genes (Pltps2, Pltps3, Pltps4 and Pltps6) showed that the expression of three genes (Pltps2, Pltps3 and Pltps4) was up-regulated, and one gene (Pltps6) was down-regulated (Table S2). Quantitative PCR quantifications of these genes in the P. lunatus plants validated the DGE results and showed a similar tendency in up/down-regulation of these four genes (Fig. 2; Table S2). These results correlate significantly with higher emissions of terpenoids from ALA-treated and spider mite-treated P. lunatus (P < 0.05, Student’s t-test) (Fig. S2), suggesting the involvement of the TPSs PITPS3 and PITPS4 encoded by Pltps3 and Pltps4 in DMNT and TMTT production.

Functional characterization of PITPS3 and PITPS4

Using recombinant protein of Pltps3 expressed and purified from bacterial cells, in vitro enzyme assays showed that PITPS3 was able to convert GPP to linalool, and FPP to (E)-nerolidol (Fig. 3a). Interestingly, PITPS3 was unable to convert GGPP to (E,E)-geranyllinalool. In similar assays, recombinant protein of Pltps4 was able to produce linalool, (E)-nerolidol, and (E,E)-geranyllinalool via GPP, FPP and GGPP, respectively (Fig. 3b).

Figure 1. Phylogenetic tree of terpene synthases (TPSs) including PITPS1–PITPS7 identified from lima bean, Phaseolus lunatus L., and those from other angiosperms. Alignment was performed using MUSCLE, and the tree was constructed by the neighbour joining method with 1,000 replications for bootstrapping. Numbers given at branches are bootstrap values. Trees were visualized using the MEGA 5.05 program. The distance reflects the proportion of amino acid sites at which two sequences are different. TPSs of P. lunatus are indicated in red. The GenBank accession numbers are listed in Table S3. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 2. Relative expression levels (mean ± SD log fold change) of candidate terpene synthase genes Pltps in lima bean, P. lunatus L. Black bars represent expression change at 24 h after treatments between ALA and mock–ALA treatment; grey bars represent expression change between spider mite, T. cinnabarinus, and mock treatment. Statistically significant differences between treated and mock-treated control plants are indicated by asterisks (*P < 0.05, LSD test).
Expression of Pltps3 and Pltps4 in transgenic rice

Two identified TPS genes (Pltps3 and Pltps4) were also transformed into rice plants to evaluate their function in vivo and ecological potential. The expression of Pltps3 and Pltps4 genes in transgenic rice were confirmed by qPCR (Fig. S3). Wild-type (WT) rice O. sativa emitted only small amounts of linalool, with no indication of DMNT and TMTT production (Fig. 4; Figs S4 and S5). Significantly higher emissions of linalool were detected in the volatiles of transgenic rice line OSPT3 expressing Pltps3 (Figs S4 and S5). Enantioselective GC analysis of collected volatiles from transgenic plants confirmed that the stereochemistry of the emitted linalool produced was (S)- (Fig. S6). DMNT was also detected from the OSPT3 rice lines, but there was no detection of TMTT. All three compounds ((S)-linalool, DMNT and TMTT) were detected in the volatiles of transgenic plant OSPT4 expressing Pltps4 (Figs S3 and S4) (P < 0.05, LSD test). Furthermore, levels of TMTT in the volatiles of OSPT4 were significantly higher than levels found in the volatiles from OSPT3 and WT plants (P < 0.05, LSD test) (Fig. 4).

Attractiveness of transgenic rice plants to parasitic wasps, C. chilonis

Female C. chilonis wasps were attracted significantly to the OSPT3 and OSPT4 rice lines expressing Pltps3 and Pltps4 genes than to the WT (ZH11 variety) plants (OSPT3 line versus WT plant: χ² = 44.78, P < 0.01; OSPT4 line versus WT plant: χ² = 28.75, P < 0.01) (Fig. 5a). Similarly, wasps preferred volatiles collected from OSPT3 and OSPT4 rice lines compared to the volatiles collected from WT plants (OSPT3 volatiles versus WT volatiles: χ² = 21.35, P < 0.01).
**DISCUSSION**

In the current study, new TPS genes responsible for the production of volatile defence terpenoids in lima bean, *P. lunatus*, were identified and characterized, and successfully expressed in rice by genetic engineering. The released defence terpenoids from transgenic rice plants enhanced the attraction of the parasitic wasp *C. chilonis*, a natural enemy of striped rice borers, *C. suppressalis*, in laboratory behavioural experiments.

Of the seven TPSs identified, three TPSs (PITPS1, PITPS5 and PITPS7) belong to the TPS-b subfamily. PITPS5, along with possibly PITPS1 and PITPS7, could function as a (E)-caryophyllene synthase because of its homology to a (E)-caryophyllene synthase characterized from the common grape vine, *V. vinifera* (Martin et al. 2010). The transcript encoding for PITPS5 was also found up-regulated in the DGE analysis (Table S2). However, the function of these putative TPSs needs to be verified by further enzyme assays with recombinant proteins.

The other four PITPSs (PITPS2, PITPS3, PITPS4 and PITPS6) were clustered into the TPS-g subfamily, which are characterized by the production of acyclic terpenes, linalool and (E)-nerolidol (Yuan et al. 2008; Chen et al. 2011; Green et al. 2011). The transcripts Pltsp2, Pltsp3 and Pltsp4 were up-regulated by spider mite infestation. PITPS2 was previously reported to be a linalool/(E)-nerolidol/(E,E)-geranyllinalool synthase, which is involved in TMTT biosynthesis (Brillada et al. 2013). This supports our data that the newly identified TPS genes (Pltsp3 and Pltsp4) are also involved in the biosynthesis of homoterpenes and able to catalyse the conversion of GPP to linalool and FPP to (E)-nerolidol and, for PITPS4, GGPP to (E,E)-geranyllinalool. Demonstration of these in vitro enzyme activities indicates that PITPS2, PITPS3 and PITPS4 are functionally conserved in *P. lunatus*. Interestingly, although PITPS2 and PITPS4 share similar substrate specificity, they have a low homology (48%). Although PITPS3 shares 39% similarity in amino acid sequence with PITPS2, it could not convert GGPP to (E,E)-geranyllinalool. Transgenic rice plants OSPT3 expressing Pltsp3 did not emit TMTT. This suggests that PITPS3 can discriminate GGPP from FPP and FPP, and is a specific TPS involved in DMNT biosynthesis in *P. lunatus*. Furthermore, PITPS3 is, from a phylogenetic perspective, an ancestor monoligene member in the TPS-g subfamily. Thus, DMNT and TMTT biosynthesis pathways in *P. lunatus* as a defence response against herbivores may have evolved differently.

It has been shown that (E)-nerolidol and (E,E)-geranyllinalool can be converted to DMNT and TMTT via an oxidative C=C bond cleavage reaction in transgenic *A. thaliana* plants (Kappers et al. 2005; Herde et al. 2008), transgenic *L. japonica* plants (Brillada et al. 2013) and in other plants (Donath and Boland 1994; Donath and Boland 1995; Piel et al. 1998). Our functional characterization using transgenic rice plants provides evidence that PITPS3 and PITPS4, together with previous identified PITPS2 could contribute, as TPSs, to the biosynthesis of DMNT and TMTT in planta. We predicted putative plastid targeting sequences on the N-terminus of these enzymes with Distill (http://distill.ucd.ie/distill/) and Targetp (http://www.cbs.dtu.dk/services/TargetP/). Distill predicts that both PITPS3 and PITPS4 could be located in chloroplasts with high and medium confidence, respectively, while Targetp predicts that PITPS3 could be located in chloroplasts with a reliability class of 1, indicating the strongest prediction. It is usually assumed that TPSs can only access FPP if expressed in the cytoplasm, and GPP (or GGPP) if expressed into the plastids, although transgenic expression of a sesquiterpene synthase, (E)-β-farnesene (EβF) synthase, in wheat with a plastidal targeting sequence gave high yields of pure EβF in the field (Bruce et al. 2015). Furthermore, a recent review reports that GPP could also be transported from the plastid to the cytoplasm which could be accessed by the lima bean TPSs in the transgenic rice plants (Sun et al. 2016). It is also possible that these TPSs could be dually located in both the
cytoplasm and plastid to access FPP and GPP (i.e. these TPSs, after synthesis in the cytoplasm, could access FPP before they are transported into the plastid). These provide the basis for our observation that the overexpression of Pltps3 and Pltps4 in rice resulted in the increased emission of both linalool and DMNT. It has been shown that homoterpene biosynthesis can arise from different biosynthetic precursors in a tissue-specific manner (Sohrabi et al. 2015). It is critical for further studies to experimentally determine tissue specificity and cellular location of PITPS3 and PITPS4, and thus rationalize their respective substrate specificities, and examine how the conversion from FPP and GPP to (E)-nerolidol and (S)-linalool could occur in the transgenic rice plants.

There was no difference in the olfactory responses of the parasitic wasp C. chilonis to the rice transgenic lines OSPT3 and OSPT4 overexpressing Pltps3 and Pltps4, respectively. Attraction of C. chilonis to the odour of transgenic plants, headspace samples, synthetic volatile blends and the homoterpenes demonstrated that transgenic plants expressing TPSs could contribute to the recruitment of beneficial natural enemies for conservation biological control.

Defence terpenes isolated from lima bean P. lunatus following ALA treatment were dominated by the homoterpenes DMNT and TMTT, whereas spider mite T. cinnabarinus infestation resulted in emission of (E)-ocimene, (S)-linalool and (E)-caryophyllene in addition to the homoterpenes. (E)-Ocimene was not detected in rice volatiles in the current study as reported by other studies. Further studies are required to elucidate the biological and ecological significances between these treatments in the biosynthesis of the plant monoterpenoids, which has been shown to play an important ecological role in other plant–herbivore–natural enemy interactions.

In summary, this study demonstrates that the enhanced production of defence volatiles in plant by means of stable genetic engineering has the potential for biological control of herbivores through natural enemy recruitment. Further work is required to determine if there are negative phenotypic differences between transformed and non-transformed lines, and to test the concept of transgenic rice plants emitting terpenoids under field conditions for the exploitation of natural enemy parasitic wasps in order to control pest populations e.g. for control of the rice brown planthopper Nilaparvata lugens (Lou et al. 2005; Xiao et al. 2012). Recent work on genetically engineered wheat expressing the aphid alarm pheromone, (E)-β-farnesene, has shown that inducible production of the engineered TPS pathway may be required for successful recruitment of natural enemies the parasitic wasp Aphidius ervi (Bruce et al. 2015). Thus, future work on transgenic rice may need to consider inducible production of the volatile defence terpenoids for management of rice stemborers and other rice pests.

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SUPPORTING INFORMATION

Table S3. Accession numbers of protein sequences used for phylogenetic analysis of plant TPSs.

Fig. S1. Gas chromatography (GC) analysis of volatiles collected from lima bean, Phaseolus lunatus L. following (a) ALA treatment, (b) ALA mock treatment, (c) spider mite treatment for 24 h and (d) mock spider mite treatment. Peaks: 1, 1-octen-3-ol; 2, (E)-ocimene; 3, linalool; 4, DMNT; 5, (E)-caryophyllene; 6, TMTT.

Fig. S2. Emission of the homoterpenes DMNT and TMTT in lima bean, Phaseolus lunatus L. following treatment with ALA, spider mites and mock treatments. Data represent the mean ± SE quantity emitted per gram fresh weight plant material per hour collection (N = 3–6 collections per treatment). Statistical significance between treated and mock plants is indicated by asterisks (*P < 0.05, Student’s t-test).

Fig. S3. Mean (± SD) relative mRNA levels of PlTPS3 and PlTPS4 in leaves of transgenic rice lines OSPT3 and OSPT4 Oryza sativa L. ssp. japonica, variety Zhonghua 11 (ZH11). Actin was selected as the internal control gene. The expression value of PlTPS3 was set to 1. WT = wild-type rice plant; OSPT3 = PITPS3 transgenic rice line; OSPT4 = PITPS4 transgenic rice lines. Primers are listed in Table S2.

Fig. S4. GC analysis of volatiles collected from OSPT3 and OSPT4 transgenic rice lines expressing PITPS3 or PITPS4. 1 = linalool; 2 = DMNT.

Fig. S5. GC analysis of volatiles collected from OSPT3 and OSPT4 transgenic rice lines expressing PITPS3 or PITPS4. 1 = TMTT.

Fig. S6. Enantioselective GC analysis of linalool produced by transgenic rice plants and comparison of GC retention time with (S)-linalool.