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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 PITPS3 positioned at the base of this cluster. Recombinant PITPS3 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 PITPS4 showed a different substrate specificity and produced linalool and (E)-nerolidol, as well as (E,E)-geranylgeranyl 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 striped rice stemborer, *Chilo suppressalis*, were significantly attracted to the transgenic plants and their volatiles. We further confirmed this with synthetic blends mimicking natural rice volatile composition. Our study demonstrates that the transformation of rice to produce volatile terpenoids has the potential to enhance plant indirect defence through natural enemy recruitment.

Key-words: (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT); parasitic wasp; transgenic rice.

INTRODUCTION

Terpenes are synthesized in plants by terpene synthases (TPSs) essentially through two pathways, the mevalonate (MVA)

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pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, in the cytosol/endoplasmic reticulum and plastids, respectively (McGarvey & Croteau 1995). Based on phylogeny and functional studies, TPSs are commonly divided into seven subfamilies: TPS-a, TPS-b, TPS-c, TPS-d, TPS-e/f, TPS-g and TPS-h. Most monoterpene synthases belong to the TPS-b and TPS-g subfamilies (Chen, Tholl, Bohlmann & Pichersky 2011). Many TPSs are multiproduct enzymes, which catalyse the formation of multiple products from acyclic substrate molecules such as geranyl diphosphate (GPP, a precursor of monoterpenoids), farnesyl diphosphate (FPP, a precursor of sesquiterpenoids) and geranylgeranyl diphosphate (GGPP, a precursor of diterpenoids). Several multifunctional TPSs have been identified in the biosynthesis of homoterpenes (also known as tetranorterenes), such as the C₁₁ homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and the C₁₆ homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). MtTPS3 in barrelclover, *Medicago truncatula* Gaertn and PITPS2 from lima bean, *Phaseolus lunatus* L. are multifunctional enzymes that catalyse the production of linalool, (E)-nerolidol and (E,E)-geranylgeranyl from GPP, FPP and GGPP, respectively. TMTT is then produced from GGPP (Brillada et al. 2013). A cytochrome P450 enzyme, CYP82G1, has been isolated from *Arabidopsis thaliana* and shown to be involved in the latter steps of DMNT and TMTT biosynthesis from (E)-nerolidol and (E,E)-geranylgeranyl respectively (Lee et al. 2010; Tholl et al. 2011). To date, the involvement of any TPSs in the biosynthesis of DMNT in *P. lunatus* is unknown.

DMNT is emitted by many plant species following herbivore damage 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);

Pickett and Khan 2016) and that egg deposition by stemborer moths, *Chilo partellus*, on maize landrace varieties caused emission of herbivore-induced plant volatiles that attract parasitic wasps, not only egg parasitoids *Trichogramma bournieri* but also larval parasitoids *C. sesamiae* (Tamiru et al. 2011). There is no previous report that DMNT and/or linalool recruits the parasitic wasp *C. chilonis* Munukata, a natural enemy of the striped rice stemborer, *C. suppressalis* Walker.

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; Brillada 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 Pltps2 in *Lotus japonicus* enhanced the capability of transgenic plants in attracting predatory mites (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 to attract EPN, *Heterorhabditis megidis* (Poinar), for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Degenhardt et al. 2009).

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 synthases 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 ($R^2 = 0.9991$), DMNT ($R^2 = 0.9958$) and TMTT ($R^2 = 0.9910$). Differences in volatile content between samples were analysed with analysis of variance (ANOVA). For enantioselective GC analysis of (S)-linalool, an Astec® CHIRALDEX® B-DM capillary GC column (50 m \times 0.25 mm, d_f 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 DESeq 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 \log_2 (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 (Wstrand 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/EcoR I restriction enzymes sites at the 5'⁰/₃⁰ 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-PITPS3 and pET30a-PITPS4. 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.

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 MOPSO, 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-EPS 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 (χ^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. cinnabarinus* 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 1e2 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 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

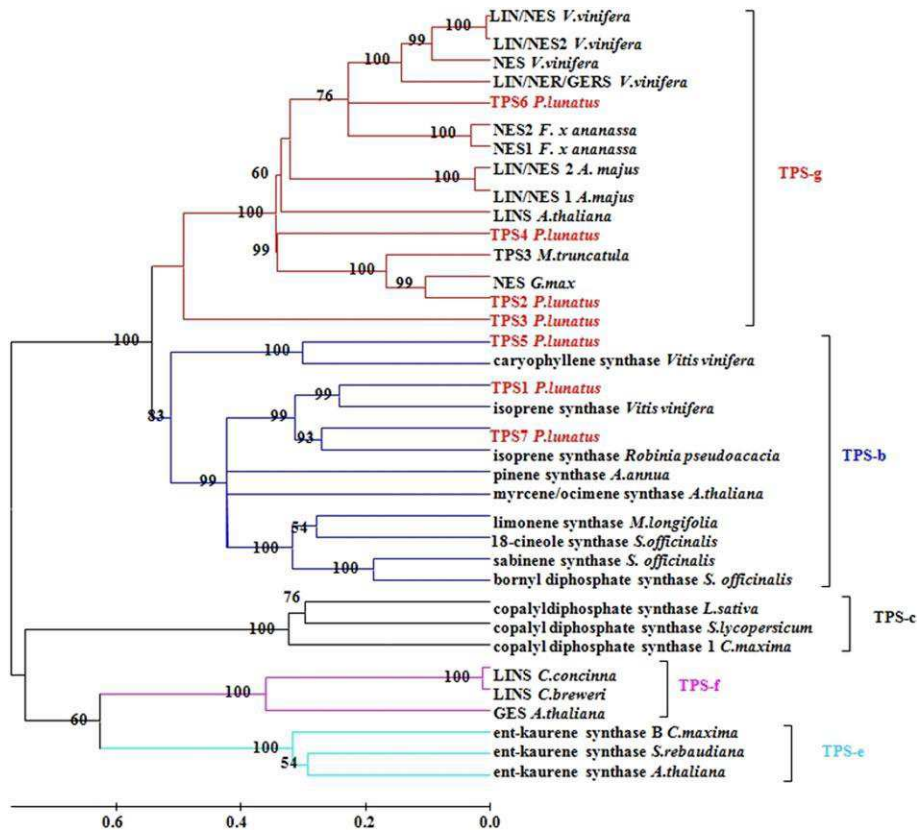


Figure 1. Phylogenetic tree of terpene synthases (TPSs) including PITPS1–PITPS7 identified from lima bean, *Phaseolus lunatus* L., and those from other angiosperms. Alignment was performed using MUSCLE, and the tree was constructed by the neighbour joining method with 1,000 replications for bootstrapping. 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]

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)-geranylinalool. In similar assays, recombinant protein

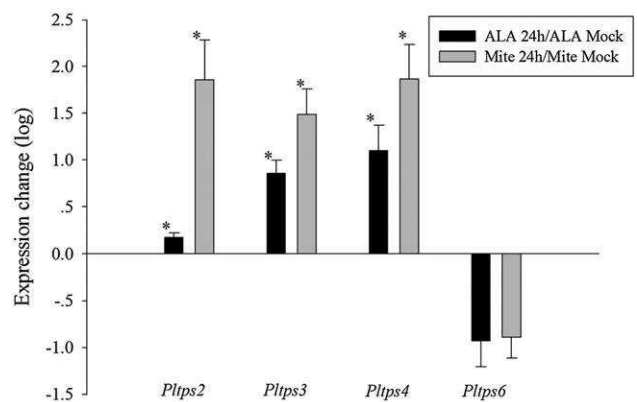


Figure 2. Relative expression levels (mean \pm SD log fold change) of candidate terpene synthase genes Pltps in lima bean, *P. lunatus* L. Black bars represent expression change at 24 h after treatments between ALA and mock-ALA treatment; grey bars represent expression change between spider mite, *T.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

of Pltps4 was able to produce linalool, (E)-nerolidol, and (E, E)-geranylinalool via GPP, FPP and GGPP, respectively (Fig. 3b).

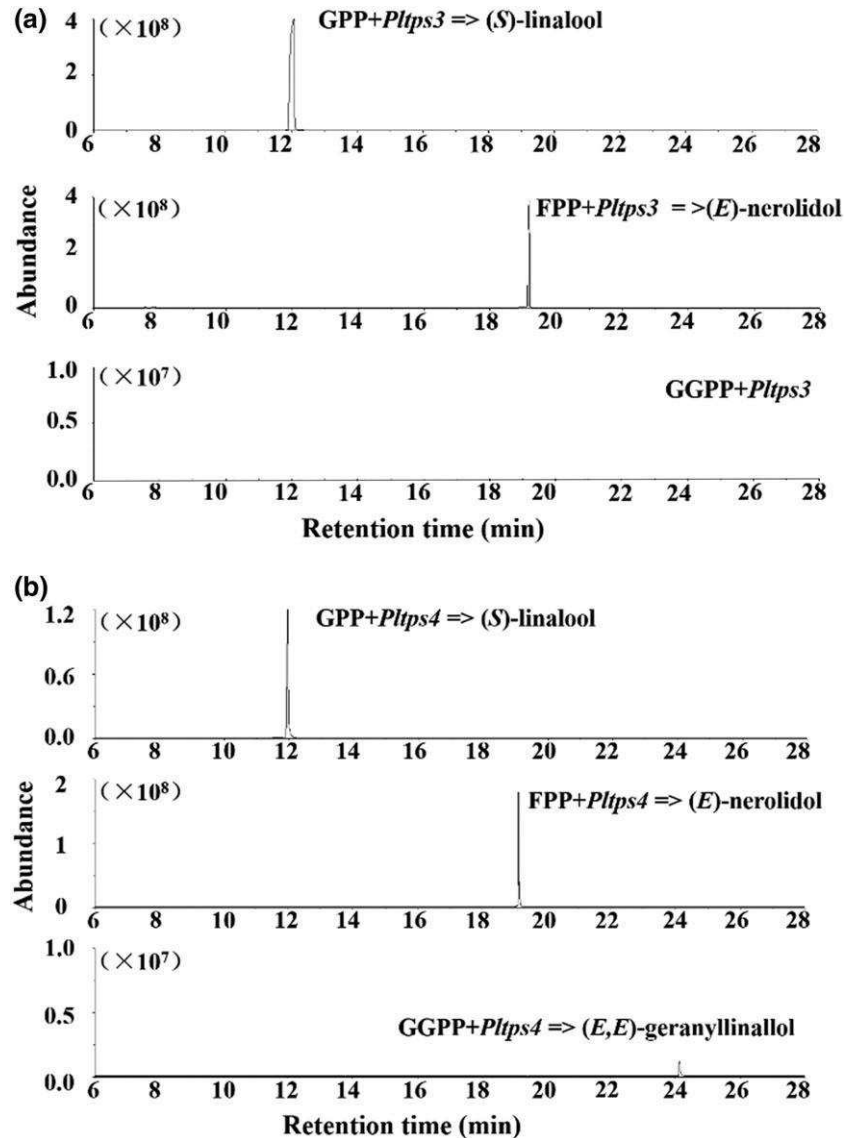


Figure 3. GC–MS analysis of the volatile products produced by recombinant PITPS3 and PITPS4 protein expressed in bacterial cells, *E. coli*. The volatile profiles of PITPS3 (A) and PITPS4 (B) are shown after the incubation with GPP (top), FPP (middle) and GGPP (bottom).

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: $\chi^2 = 44.78$, $P < 0.01$; OSPT4 line versus WT plant: $\chi^2 = 28.75$, $P < 0.01$) (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: $\chi^2 = 21.35$, $P < 0.01$;

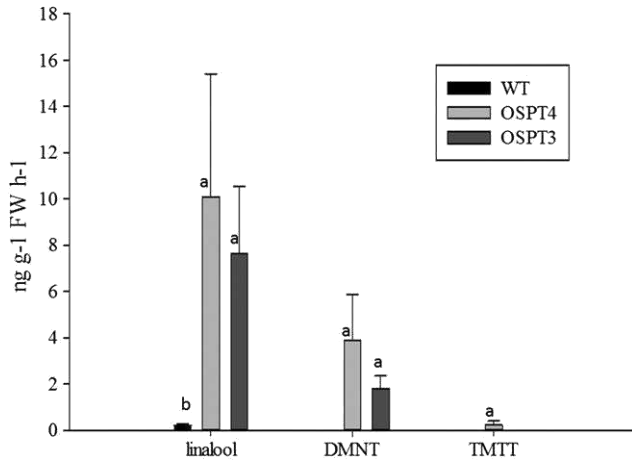


Figure 4. Emission of linalool, DMNT and TMTT from transgenic rice lines OSPT3, OSPT4 and WT rice plants, *Oryza. sativa* L. ssp. japonica, variety Zhonghua 11 (ZH11). Data are presented as the mean (\pm SD) quantity (nanograms) emitted per gram fresh weight (FW) plant material per hour. Means with different letters are significantly different ($P < 0.05$, LSD test, $N = 4$ collections per plant type).

OSPT4 volatiles versus WT volatiles: $\chi^2 = 25.81$, $P < 0.01$;

OSPT4 volatiles versus WT volatiles: $\chi^2 = 25.81$, $P < 0.01$). It was found that the wasps also preferred synthetic blends that mimicked natural blends of OSPT3 and OSPT4 volatiles (Fig. S1) compared to synthetic blends of WT volatiles (OSPT3 synthetic blends versus WT synthetic blends:

$\chi^2 = 18.38$, $P < 0.01$; OSPT4 synthetic blends versus WT synthetic blends: $\chi^2 = 29.39$, $P < 0.01$) (Fig. 5b). Wasps also significantly preferred pure DMNT and TMTT compared

with solvent controls (DMNT versus solvent control:

$\chi^2 = 37.80$, $P < 0.01$; TMTT versus solvent control:

$\chi^2 = 41.09$, $P < 0.01$), and linalool only at the highest dose tested, i.e. 1

μg (linalool versus solvent control: $\chi^2 = 7.81$, $P < 0.01$) (Fig. 5).

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 stemborers, *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)-geranylinalool 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)-geranylinalool. 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)-geranylinalool. Transgenic rice plants OSPT3 expressing *Pltsp3* did not emit TMTT. This suggests that PITPS3 can discriminate GGPP from GPP and FPP, and is a specific TPS involved in DMNT biosynthesis in *P. lunatus*. Furthermore, PITPS3 is, from a phylogenetic perspective, an ancestor monolineage 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)-geranylinalool 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 (E β F) synthase, in wheat with a plastidal targeting sequence gave high yields of pure E β F 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

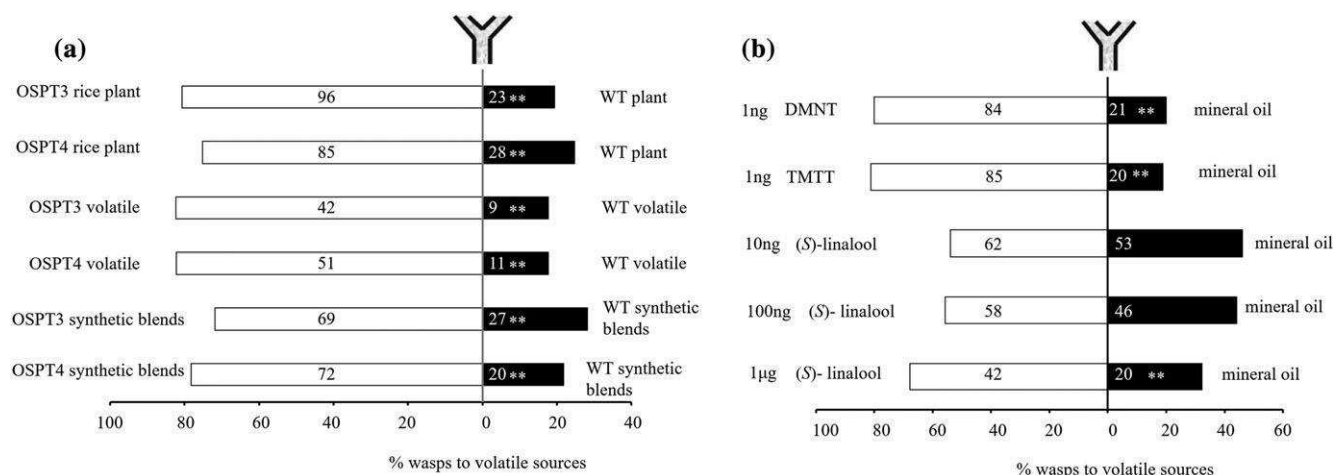


Figure 5. Behavioural responses of the female parasitic wasps, *C. chilonis* (Hymenoptera: Braconidae), in a Y-tube olfactometer. (a) Responses of *C. chilonis* to volatiles released by transgenic rice lines OSPT3 and OSPT4, collected volatiles and synthetic chemical blends. (b) Responses of *C. chilonis* to authentic standards of linalool, DMNT and TMTT. Numbers in bars represent the total numbers of wasps responding to stimuli. The bars show the percentage of *C. chilonis* responding to the stimuli. Data were analysed using χ^2 analysis to evaluate differences in each choice experiment (** $P < 0.01$).

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 stem borers 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 (\pm SD) relative mRNA levels of PITPS3 and PITPS4 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 PITPS3 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.