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Metabolic engineering of the C₁₆ homoterpene TMTT in *Lotus japonicus* through overexpression of *(E,E)-geranyllinalool synthase* attracts generalist and specialist predators in different manners

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Keywords: herbivore-induced plant volatiles (HIPVs), indirect defense, lima bean, mite, terpene, \((E,E)-4,8,12\text{-trimethyltrideca-1,3,7,11-tetraene (TMTT)}\)
Summary

- Plant defenses against herbivores include the emission of specific blends of volatiles, which enable plants to attract natural enemies of herbivores.
- Transgenic \textit{Lotus japonicus} and \textit{Nicotiana tabacum} plants, expressing \textit{PlTPS2} or its homologue \textit{Medicago truncatula} TPS3 (\textit{MtTPS3}), were produced and used for bioassays with herbivorous and predatory mites. Transgenic \textit{L. japonicus} plants expressing \textit{PlTPS2} produced (\textit{E},\textit{E})-geranyllinalool and TMTT, whereas wild-type plants and transgenic plants expressing \textit{MtTPS3} did not. Transgenic \textit{N. tabacum} expressing \textit{PlTPS2} produced (\textit{E},\textit{E})-geranyllinalool but not TMTT. Moreover, in olfactory assays, the generalist predatory mite \textit{Neoseiulus californicus} but not the specialist \textit{Phytoseiulus persimilis} was attracted to uninfested, transgenic \textit{L. japonicus} plants expressing \textit{PlTPS2} over wild-type plants. The specialist \textit{P. persimilis} was more strongly attracted by the transgenic plants infested with spider mites than by infested wild-type plants.
- Predator responses to transgenic plant volatile TMTT depend on various background volatiles endogenously produced by the transgenic plants. Therefore, the manipulation of TMTT is an ideal platform for pest control via the attraction of generalist and specialist predators in different manners.
Indirect defenses of plants against herbivores include the emission of specific blends of volatiles in response to herbivory (HIPVs, herbivore-induced plant volatiles), which enables the plants to attract carnivorous natural enemies of herbivores (Arimura et al., 2009; Maffei et al., 2011). Volatile terpenoids are the major products among HIPVs, and in legumes include monoterpenes (C\textsubscript{10}), sesquiterpenes (C\textsubscript{15}), and tetranor-terpenoids (homoterpenes, (E)-4,8-dimethyl-1,3,7-nonatriene [DMNT, C\textsubscript{11}] or (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [TMTT, C\textsubscript{16}]) (Ozawa et al., 2000; Leitner et al., 2005). The molecular diversity of terpenes is expanded as a result of the use by terpene synthases (TPSs) of different prenyl diphosphates as substrates, with these prenyl diphosphates being derived from the mevalonate (MVA) pathway in the cytosol/endoplasmic reticulum or the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway in plastids (Lange et al., 2000). TPSs are often multi-product enzymes, and thus even a single TPS can contribute significantly to the plasticity of blends, especially blends produced in response to herbivory (Köllner et al., 2004).

In addition, during the last decade several other types of multi-functional TPSs have been studied, e.g., in Medicago truncatula, MtTPS3, which encodes a multi-functional enzyme producing linalool, (E)-nerolidol (precursor of DMNT) and (E,E)-geranyllinalool (GL, precursor of TMTT) from different prenyl diphosphates serving as substrates (Arimura et al., 2008). In turn, it has been shown that the herbivore-induced biosynthesis of TMTT is catalyzed by the concerted activities of AtGES, a monofunctional enzyme producing GL (Herde et al., 2008), and CYP82G1
(Lee et al., 2010) in Arabidopsis (Arabidopsis thaliana). Of interest is the fact that
AtGES is not localized to the plastids, where diterpene synthases are primarily located,
but rather resides in the cytosol or in the endoplasmic reticulum. It is likely that the
AtGES substrate geranylgeranyl diphosphate (GGDP) is present in these compartments,
since there are two Arabidopsis GGDP synthases with a localization pattern similar to
that observed for AtGES (Okada et al., 2000). In turn, CYP82G1, a cytochrome P450
monooxygenase of the Arabidopsis CYP82 family, is responsible for the breakdown of
GL to the insect-induced TMTT. Homology-based modeling and substrate docking
support an oxidative bond cleavage of the alcohol substrate via syn-elimination of the
polar head, together with an allylic C-5 hydrogen atom (Lee et al., 2010).

The use of transgenic plants, especially, represents a novel solution to the
challenges of studying the biochemical and ecological relevance of terpenes (Aharoni et
al., 2005). For instance, targeting FaNES1, a strawberry linalool/(E)-nerolidol synthase,
to the mitochondria resulted in the production of (E)-nerolidol and DMNT in transgenic
Arabidopsis plants (Kappers et al., 2005). Based on the presence of
mitochondria-targeted farnesyl diphosphate (FDP) synthase and TPS (FaNES2, a
homologue of FaNES1) (Aharoni et al., 2004), it was suggested that this cell
compartment might also contain a potential pool for sesquiterpene biosynthesis.
Transgenic plant approaches using TPSs are therefore useful to reveal novel
biosynthetic pathways of terpenes and deepen our understanding of their mechanisms.
Moreover, such manipulations of volatile blends may also be applicable in integrated
pest management strategies that employ volatiles attracting herbivore enemies in
so-called push–pull systems (Khan et al., 2008). All these considerations taken together indicate that genetic “gain” of particular terpenes is essential to fully exert the ecological and agricultural functions of individual terpenes.

The spider mite *Tetranychus urticae* is a serious pest of agricultural, vegetable, fruit and ornamental plants (Helle & Sabelis, 1985). *T. urticae*-induced plant volatiles enhance the prey-searching efficacy of predatory mites, and this attraction results in the extermination of *T. urticae* from the plants (Helle & Sabelis, 1985). There is some evidence that lima beans respond to feeding spider mites by emitting herbivore-induced plant volatiles (including TMTT) to attract the specialist predatory mite *Phytoseiulus persimilis* (van Wijk et al., 2008) and the generalist predatory mite *Neoseiulus californicus* (Shimoda, 2010). Our previous study showed the ability of the lima bean (*E*)-β-ocimene synthase gene to enhance the attraction of predatory mites (*P. persimilis*) (Shimoda et al., 2012). It should, however, be kept in mind that the host plant strategy to resist spider mites is not based only on single volatile compounds, but rather on a mixture of them. This can be accomplished by the concerted action of different genes or by the harmonized activity of some multifunctional genes. Accordingly, plant species have a multi-gene family of TPSs (Degenhardt et al., 2009), and it is necessary to understand which (and how) genes are involved in plant-mite interactions. In the current study, we isolated a lima bean TPS cDNA (*PlTPS2*) and identified the gene product as a terpene synthase of the diterpene alcohol GL, a precursor of TMTT that was predicted to be an airborne infochemical in ecosystems. By assessing the nature of this lima bean terpene synthase in transgenic *Lotus japonicus* plants expressing it, we identified a
critical role of PITPS2 in the regulation of herbivore-induced formation of GL and TMTT.

This paper also addresses the issue of the complicated nature of indirect plant defenses when transgenic plants are used in indirect pest control.

Materials and Methods

Plants and arthropods

Lima bean (*Phaseolus lunatus*) plants were grown in a greenhouse. Each individual plant was grown in a plastic pot in a growth chamber at 25°C with a photoperiod of 16 h (natural+supplemental light) for 2 weeks. Tobacco (*Nicotiana tabacum* cv. SR1) and *Lotus japonicus* (ecotype Miyakojima MG-20) plants were grown in plastic pots in a growth chamber at 25°C (16 h photoperiod at a light intensity of 80 µE m⁻² s⁻¹) for 4-6 weeks. *T. urticae* was reared on kidney bean plants (*Phaseolus vulgaris*) in another greenhouse under the same conditions. *P. persimilis* was obtained from a commercial source (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands). *N. californicus* was collected from *Pueraria lobata* plants infested with *Tetranychus pueraricola* in a field at the National Agricultural Research Center in Ibaraki Prefecture, Japan. These predators were reared on *T. urticae*-infested bean plants in a climate-controlled room (25°C, 16 h photoperiod). Fertilized adult females 3–5 d after the final molting were used for the bioassays. To prepare starved predators, the predators were individually placed in sealed plastic tubes (1.5 ml), each containing a drop of water (3 µl), in the laboratory for 24 h.
For chemical treatment, JA (0.5 mM, pH 5.8–6.0, Wako Pure Chemical Industrials, Ltd., Osaka, Japan) in 2 mL of water was sprayed onto intact plants in plastic pots. Alamethicin (0.1 µM, Sigma-Aldrich, St. Louis, MO, USA) was applied to the petioles of detached lima bean plantlets in aqueous solution. For herbivore treatment, a lima bean plant and an *L. japonicus* plant were treated with 40 or 50 *T. urticae* adult females, respectively. All treatments were carried out in a climate-controlled chamber at 25°C (16 h photoperiod).

cDNA cloning

Total RNA was isolated and purified from leaf tissues using a Qiagen RNeasy Plant Mini Kit and an RNase-Free DNase Set (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA, USA), oligo(dT)$_{12-18}$ primer, and 1 µg of total RNA at 50°C for 50 min. For polymerase chain reaction (PCR), primers for the *PlTPS2* cDNA fragment were designed using partial DNA sequences of an expressed sequence tag (EST) clone (annotation number: CV540470) obtained from the TIGR *P. vulgaris* EST database: http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=p_vulgaris. PCR was performed with 2 min at 95°C; 35 cycles of 15 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C. Further cloning of 5’- and 3’-ends was accomplished by rapid amplification of cDNA ends (RACE) PCR using a First Choice RLM-RACE Kit (Ambion, Austin, TX, USA) following the manufacturer’s protocol.
Recombinant PlTPS2 enzyme preparation and assay

For functional identification, cDNAs were amplified by PCR using Pfu DNA Polymerase (Promega, Madison, WI, USA) with a set of primers for an open reading frame (ORF) of PlTPS2. The cDNA was subcloned into the pHis8-3 expression vector (Jez et al., 2000). The recombinant vectors (pHis8.3-PlTPS2) were transformed into Escherichia coli BL21-CodonPlus(DE3). The resultant bacterial strain was grown to an A$_{600}$ = 0.5 at 37°C in 5 ml of LB medium with kanamycin at 50 µg ml$^{-1}$. Cultures were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and kept overnight at 16 °C while being shaken at 200 rpm. Cells were pelleted by centrifugation and resuspended in 250 µl of assay buffer (25 mM HEPES, pH 7.3, 12.5 mM MgCl$_2$, 0.25 mM MnCl$_2$, 0.25 mM NaWO$_4$, 0.125 mM NaF, 10 mM DTT, 10% glycerol). Resuspended cells were broken by sonication. Cell extracts were clarified by centrifugation and assayed for TPS activity with 50 µM geranyl diphosphate (GDP, Echelon Biosciences Incorporated, Salt Lake City, UT, USA), FDP (Echelon Biosciences Incorporated) or GGDP (Sigma-Aldrich). The assay mixture was covered with pentane containing n-bromodecane (100 ng µl$^{-1}$), as an internal standard, to trap volatile products. After incubation at 30°C for 1 h, the pentane layer was transferred to a glass vial and analyzed. Extracts of E. coli transformed with expression vectors without the TPS gene were used as controls following the above procedure. The enzymatic reaction products were analyzed on a ThermoQuest/Finnigan TRACE GC 2000 with a TRACE MS (Manchester, UK) equipped with an EC$^{TM}$-5 capillary column (0.25 mm i.d.
x 15 m with 0.25-mm film, Alltech, Deerfield, IL, USA). Injection volume: 1 µl; split 1:100; 220°C. Ionization energy: 70 eV. Compounds were eluted under programmed conditions starting from 40°C (2-min hold) and ramped up at 10°C min\(^{-1}\) to 200°C followed by 30°C min\(^{-1}\) to 280°C, which was held for 1 min prior to cooling. Helium at a flow rate of 1.5 ml min\(^{-1}\) served as a carrier gas. The products were identified and quantified as described previously (Arimura et al., 2008).

Generation of transgenic *L. japonicus* and *N. tabacum* plants

The full-length coding region of lima bean *PlTPS2* (GenBank accession no. KC012520) or *MtTPS3* (AY766249) was inserted into binary vector pMDC32 using the Gateway cloning system (Invitrogen). The resulting plasmid, pMDC32-*PlTPS2* or pMDC32-*MtTPS3*, was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Tobacco plants that had been aseptically grown from seeds for about 1 month were transformed via an *A. tumefaciens*-mediated leaf disc procedure (Horsch et al., 1985). *L. japonicus* was also transformed using the *A. tumefaciens*-mediated transformation procedure described by Imaizumi et al. (2005). As the selection agent, 30 mg l\(^{-1}\) or 50 mg l\(^{-1}\) hygromycin was used for *N. tabacum* or *L. japonicus*, respectively. After rooting and acclimatization, the regenerated plants were grown in a closed greenhouse to set seeds. About 8 lines of transgenic T\(_1\) seeds from each transformant were tested for germination on 1/2 Murashige and Skoog medium supplemented with 20-30 mg l\(^{-1}\) hygromycin. T\(_2\) seeds harvested from each individual T\(_1\) plant that showed ca. 3:1 segregation ratio were tested for hygromycin-resistance...
again. Both $T_1$ and $T_2$ plant lines were used for further chemical and gene expression analyses, and homozygous $T_3$ plant lines were used in bioassays. A homozygous *L. japonicas* line transformed with the binary plasmid pIG121Hm, expressing hygromycin phosphotransferase (*hpt*) and intron-containing β-glucuronidase [GUS] genes (Hiei *et al.*, 1994), was used as a control.

Quantitative reverse transcription (RT)-PCR

Total RNA was isolated from leaf tissues using a Qiagen RNeasy Plant Mini Kit and an RNase-Free DNase Set (Qiagen) following the manufacturer’s protocol. First-strand cDNA was synthesized using a PrimeScript RT reagent Kit (Takara, Otsu, Japan), and 0.5 µg of total RNA at 37°C for 15 min. Real-time PCR was performed on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using FastStart Universal SYBR Green Master (ROX) (Roche Applied Science, Indianapolis, IN, USA), cDNA (1 µl from 10 µl of each RT product pool), and 300 nM primers. The following protocol was used: initial polymerase activation: 10 min at 95°C; then 40 cycles of 15 s at 95°C and 60 s at 60°C. PCR conditions were chosen by comparing threshold values in a dilution series of the RT product, followed by a non-RT template control and non-template control for each primer pair. Relative RNA levels were calibrated and normalized with the level of *PlACT1* mRNA (GenBank accession no. DQ159907), *LjTUB* (AB510590) or *NtACT* (GQ281246). Primers used for this study are shown in Table S1.
Gateway Technology (Invitrogen) was used for the generation of pGWB451-PlTPS2 transformation constructs, which consisted of PlTPS2 ORF cDNA bearing an N-terminal fusion to G3GFP under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Nakagawa et al., 2007). The resulting plasmid, pGWB451-PlTPS2, was transformed into Agrobacterium tumefaciens strain EHA101 by electroporation. pGWB452, which expresses G3GFP under the control of the CaMV 35S promoter (Nakagawa et al., 2007), served as control. The bacteria were cultured in 50 ml of Luria-Bertani (LB) medium/rifampicin/kanamycin/spectinomycin at 28°C for 36 h, and 1 ml of the cell culture was inoculated in 50 ml of LB/kanamycin/spectinomycin. Cells were harvested by centrifugation and resuspended in 10 mM MES-NaOH, pH 5.6, 10 mM MgCl₂ and 150 mM acetoxyringone (Sigma-Aldrich). The bacterial suspensions were adjusted to OD₆₀₀ 1.0, incubated for 4 h at 28°C, and then infiltrated into leaves of 2-3-week-old lima bean plants using a needleless syringe. After 36-40 h, GFP fluorescence was observed in lima bean leaves under a Nikon Eclipse C1 spectral confocal laser scanner microscope (CLSM) with a 60x Plan Apo 1.40/oil objective (Nikon Instruments, Tokyo, Japan). The microscope operates with two lasers: GFP was excited at 488 with a krypton/argon laser, and chlorophyll autofluorescence was excited at 647 nm with a HeNe-Laser. Emissions wave was collected through a 506 to 530 nm band-pass filter (for GFP) and a 650 nm low-pass filter (for chlorophyll).
Headspace volatiles from potted plants were collected in a glass container (2 l) using 100 mg of Tenax-TA resin (20/35 mesh; GL Science, Tokyo, Japan) packed in a glass tube (3.0 mm i.d., 160 mm length) in a laboratory room (25°C, light intensity of 80 µE m⁻² s⁻¹). Pure air gas (CO < 1 ppm, CO₂ <1 ppm, THC < 1 ppm) was drawn into the glass bottle, and volatile compounds from the headspace of the bottle were collected with Tenax-TA for 2 h at a flow rate of 100 ml min⁻¹. n-Tridecane (0.1 µg) as internal standard was also added to the glass container. The volatile compounds collected were analyzed by gas chromatography-mass spectrometry (GC-MS) according to the method described by (Shimoda et al., 2012). The headspace volatiles were identified and quantified by comparing their mass spectra and retention times with those of authentic compounds. All experiments were repeated four to seven times. DMNT and TMTT were synthesized in the laboratory.

Non-volatile analysis

Leaves of 4-6-week-old *N. tabacum* (about 3 g each) or *L. japonicus* (about 0.5-1 g each) plants were harvested and ground to a fine powder with a mortar and pestle under liquid nitrogen. Ethyl acetate (1:5 w/v) spiked with 25 µg of internal standard (borneol, Sigma-Aldrich) was added to each sample, and then the mixture was homogenized. Extracts were transferred into a glass tube and centrifuged at 5000 g for 5 min at 4°C. The resultant pellets were rinsed with the same volume of ethyl acetate and centrifuged. The combined organic layers were subsequently adjusted to 500 µl under a nitrogen flow.
The extracts from *N. tabacum* and *L. japonicus* (1 µl and 5 µl, respectively) were injected into a gas chromatograph (6890N, Agilent Technologies, Santa Clara, CA, USA) coupled with a mass spectrometer (5973A, Agilent Technologies). Compounds were separated on a Zebron ZB-5MS capillary column (7HG010-11, Phenomenex, Torrance, CA, USA; stationary phase: 95% polydi-methyl siloxane - 5% diphenyl, length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 µm) with the following temperature program: 60°C for 5 min followed by a temperature rise at a 4°C min⁻¹ rate up to 270°C and 7°C min⁻¹ rate to 290°C (held for 1 min). The carrier gas was He with a constant flow of 1 ml min⁻¹, transfer line temperature to MSD was 280°C, ionization energy (EI) 70 eV, and full scan range 50–320 m/z. GL was identified by comparison with an authentic standard (Sigma-Aldrich) using the NIST mass spectral search software v2.0 with the NIST 98 library. GL quantitation was assessed by GC-FID (6890N, Agilent Technologies) with the same experimental procedures as described above.

In order to analyze terpenoid-sugar conjugates, the pellets obtained above were extracted with 4 ml of citrate buffer (pH 5.2), transferred to a glass tube and centrifuged at 5000 g for 5 min at 4°C. Supernatants (about 3 ml) were collected in fresh glass tubes and hydrolyzed enzymatically by adding 10 mg (about 60 U) of β-glucosidase (from almonds, Sigma-Aldrich). The mixture was covered with pentane (5 ml) containing borneol (25 µg), as an internal standard, to trap volatile products. After incubation at 37°C for 24 h, the pentane layer was transferred to a glass vial, reduced to a final volume of 500 µl and analyzed using GC-FID and GC-MS (described above). Octyl-β-glucoside (Carbosynth, Compton, Berkshire, UK) was used as an external
control following the above procedure.

Assay for herbivores

Adult females of *T. urticae* were individually introduced onto leaf discs, detached from wild-type (WT), GUS, LjPT3 or LjMT6 placed on water-soaked cotton wool in a Petri dish (9 cm diameter, 1.7 cm deep). Each dish contained 5 detached leaves. Following incubation in a climate-controlled room (25°C, 16 h photoperiod) for 3 days, the survivors and eggs oviposited were counted under a binocular microscope (MZ160 microscope with TL5000 Ergo light base with automatic aperture; Leica, Tokyo, Japan). Forty independent females were analyzed for each line.

Larvae of *T. urticae*, within 3 h after hatching, were individually introduced onto leaf discs. We observed the leaf discs daily and counted the number of adults under a binocular microscope under the same conditions described above. Forty independent larvae were analyzed for each line.

Ten *L. trifolii* adult females were allowed to oviposit eggs on potted LjPT3 or WT plants in a netted plastic cage (25 × 33 × 30 cm) in a climate-controlled room (see above) for 1 day. Leaves on which a single egg was inserted were collected and used for subsequent assays. We observed the leaf discs daily and counted the number of pupae emerging from the leaf tissues under the same conditions described above. Forty independent larvae were analyzed for each line.

Olfactory assay
Each olfactory bioassay was performed using a Y-tube olfactometer in a laboratory (25°C, light intensity of 80 µE m\(^{-2}\) s\(^{-1}\)), according to the method described by Shimoda et al. (2012). The odor sources used were divided into the following six types: uninfested WT plants, infested WT plants, uninfested LjPT3 plants, infested LjPT3 plants, uninfested LjPT5 plants, and infested LjPT5 plants. For infestation, a potted plant in a plastic pot was infested with *T. urticae* (50 adult females) for 2 days. For each assay, 10 intact plants (each plant weighing about 2.5 g; 5 plants/pot) were used as an odor source.

Predators were individually introduced at the start point in the olfactometer, and the numbers of predators choosing either sample or control odor sources were recorded. Predators that did not choose within 5 min (‘no choice’ subjects) were excluded from the statistical analysis. Assays using 20 predators were carried out as a single replicate in a day. Four replications (i.e. 80 predators in all) were carried out on different days. The results from three or four replications of each experiment were subjected to a replicated G-test; the pooled G-value (*G*\(_p\), df = 1 in each) was used to test the null hypothesis that the predators exhibited a 50 : 50 distribution over the sample and control odor sources in each experiment (Sokal & Rohlf, 1995). We also confirmed that there was no significant heterogeneity among replications in each experiment (df = 3, *P* > 0.05 for each *G*\(_h\), replicated G-test), suggesting good reproducibility of the two-choice test.

**Results**
Functional characterization of PITPS2

In order to verify the functional involvement of PITPS2 in volatile biosynthesis, we determined a full-length cDNA sequence for the gene from lima bean (GenBank accession no. KC012520). The deduced nucleotide sequence of PITPS2 encodes a predicted protein of 569 amino acids that shares 78% identity and 83% similarity with a Glycine max predicted (3S,6E)-nerolidol synthase (XP_003528418) and 66% identity and 72% similarity with a Medicago truncatula linalool/(E)-nerolidol/GL synthase (MtTPS3, AY766249) in the TPS-g group (Fig. S1). A functional assay of the recombinant PITPS2 with prenyl diphosphatate (GDP, FDP or GGDP) as substrate resulted in the production of the monoterpene linalool from GDP, the sesquiterpene (E)-nerolidol from FDP and the diterpene GL from GGDP (Fig. 1). The recombinant protein generated linalool as the predominant product, as well as (E)-nerolidol and GL at approximately 82% and 16% of the rate of linalool. This ratio is different from the composition of the product of the homologue MtTPS3 (linalool:(E)-nerolidol:GL = 5:100:65 (Arimura et al., 2008). A control extract prepared from the BL21-CodonPlus(DE3) strain transformed with a plasmid without the PITPS2 cDNA insert did not produce any terpene products (data not shown).

Expression of PITPS2, and formation of its products in response to fungal elicitor and spider mites

Using quantitative RT-PCR of RNA from lima bean leaves, we analyzed the transcriptional levels of PITPS2 upon external application of jasmonic acid (JA, 0.5
mM), *T. urticae* spider mite feeding, or alamethicin (ALA, 0.1 mM), an elicitor of the
plant pathogenic fungus *Trichoderma viride* (Arimura *et al*., 2008) (Fig. 2a). ALA
induced *PlTPS2* transcripts at 2 h, and more dramatically at 6 and 24 h, after application.
Similarly, *PlTPS2* transcript levels were increased gradually over the time-course of
exposure of lima bean leaves to spider mites (~24 h). In contrast to these stimuli,
however, JA application did not induce the transcript, indicating the lack of dependence
of *PlTPS2* activation on JA signaling (Fig. 2a).

Next, to test if the transcriptional profile of *PlTPS2* was reflected by the emission
of linalool and of the homoterpenes DMNT and TMTT, we measured headspace
volatiles emitted from lima bean plants exposed to spider mite feeding and from leaves
treated with JA or ALA (Fig. 2b). The oxidative degradation of *(E)-nerolidol and GL
generates DMNT and TMTT (Donath & Boland, 1994), and these volatiles have been
found in the blend of HIPVs from lima beans exposed to ALA or spider mites (Ozawa *et
al*., 2000; Engelberth *et al*., 2001). As expected, emission of DMNT and TMTT from
lima bean plants was induced, in similar manner to the *PlTPS2* transcriptional profile, by
ALA treatment or spider mite feeding. Emission of these homoterpenes, however,
remained undetectable in JA-treated plants, in contrast to the emission of linalool,
which was elevated only when JA was provided. GL, one of the *PITPS2* products, was
hardly detected in the headspace of any of the lima bean samples, whereas this diterpene
accumulated in leaves infested with spider mites or treated with ALA (Fig. 2b).

Subcellular localization of *PITPS2*
The subcellular localization of PlTPS2-GFP fusion proteins (Fig. 2c) in transiently expressing lima bean leaf cells was plastidial (Fig. 2d). This result reflected the plastidial localization of MtTPS3, a homologue of PlTPS2 (Gomez et al., 2005) but not the localization of Arabidopsis GL synthase (AtGES), which is targeted to the cytosol or the endoplasmic reticulum (Herde et al., 2008).

Transgenic plants expressing TPSs

In order to understand the physiological and ecological features of PlTPS2 and its M. truncatula homologue MtTPS3 (Arimura et al., 2008), transgenic plants constitutively expressing these genes were generated. The respective ORF sequences under the control of the CaMV 35S promoter were transformed into N. tabacum and L. japonicus, resulting in four individual lines (NtPT [N. tabacum expressing PlTPS2], NtMT [N. tabacum expressing MtTPS3], LjPT [L. japonicus expressing PlTPS2], and LjMT [L. japonicus expressing MtTPS3]). Following selection for hygromycin resistance in the T1 and T2 plant lines, positive plants were grown and used for further experiments. All the transgenic lines exhibited trans-gene (PlTPS2 or MtTPS3) expression in the leaves, whereas wild-type (WT) plants did not (Fig. S2). In addition, none of the transgenic lines exhibited any detectable differences in their morphology (Figs. S3 and S4).

None of the transgenic tobacco lines used for analysis exhibited detectable levels or increased emission of the TPS-derived volatiles (i.e., linalool, (E)-nerolidol, DMNT and TMTT; Fig. S5). The non-volatile diterpene GL was, however, accumulated at significantly higher levels in the leaves of NtPT lines than in the WT (P<0.05, Dunnett’s
test) (Fig. 3). This diterpene was very slightly elevated in NtMT lines when compared to its level in WT (NtMT3, 1.9 times; NtMT4, 1.4 times, $P > 0.05$, Dunnett’s test).

Interestingly, substantial production of both TMTT and GL was detected only in the leaves of transgenic *L. japonicus* plants expressing *PlTPS2* (LjPT3 and 5; $P < 0.05$, Dunnett’s test), whereas WT, GUS-transgenic control plants, and transgenic plants expressing *MtTPS3* (LjMT3 and 6) showed no detectable production (Fig. 4). In response to spider mite attack, both LjPT3 and LjPT5 plants emitted TMTT, at similar levels as the uninfested plants (Fig. 4b). In contrast, after infestation, the accumulation of GL decreased to 44% and 32% of that in uninfested LjPT3 and LjPT5 plants, respectively. The emission of TMTT was not observed in uninfested WT and LjMT6 plants and very slightly increased in response to spider mite attack, although GL was observed neither in uninfested nor in infested plants. DMNT was similarly emitted from infested WT, LjPT3 and LjMT6 plants (Fig. 4b).

Some terpene alcohols might be glycosylated and accumulated as non-volatiles in plant cell vacuoles (Houshyani et al., 2013). Therefore, we also checked for the presence of glycosylated forms of linalool and GL. No glycosylated compounds were, however, detected in any of the transgenic lines analyzed.

Resistance of transgenic *L. japonicus* plants to arthropod herbivores

We evaluated the effects of transgenic plant products on survival, oviposition and development of pest herbivores. *T. urticae* females survived and reproduced equivalently among WT, GUS, LjPT3, and LjMT6 lines (survival: $\chi^2 = 2.888$, $df = 3$, $P$
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Moreover, no significant difference was observed among those plants in the development of the next generation of *T. urticae* (survival ratio from larva to adult: $\chi^2 = 1.027, df = 3, P = 0.795$, GLM-test, Fig. 5c). We evaluated another herbivore species, the American serpentine leafminer *L. trifolii*, and found that its larvae developed similarly between WT and LjPT3 (survival ratio from larval to pupal stages: $\chi^2 = 3.127, df = 1, P = 0.077$, GLM-test, Fig. S6).

Olfactory response of transgenic plants

We assessed the influence of HIPVs and the trans-volatile TMTT on the olfactory responses of *N. californicus* females. *N. californicus* is a generalist feeder that can exploit various foods such as small insects and pollen, as well as species of the genus *Tetranychus* (Shimoda, 2010). The predators showed significant preferences for HIPVs from *T. urticae*-infested WT plants of *L. japonicus* ($G_p = 4.312, P < 0.05$, replicated G-test, Fig. 6a) and TMTT from uninfested LjPT3 plants ($G_p = 7.312, P < 0.01$, replicated G-test), in comparison to basal volatiles from uninfested WT plants. However, the predators did not discriminate between infested LjPT3 (HIPVs + TMTT, see Fig. 4) and infested WT plants (HIPVs) ($G_p = 0.450, P = 0.502$, replicated G-test), indicating that TMTT had no additive effect on the attractiveness of HIPVs for *N. californicus*.

We next assessed olfactory responses of *P. persimilis* females. *P. persimilis* is a specialist predator that needs abundant *Tetranychus* spider mites as prey (Walzer *et al.*, 2001; Shimoda *et al.*, 2012). The predators did not discriminate TMTT from uninfested...
LjPT3 plants from basal volatiles from uninfested WT plants ($G_p = 1.028, P = 0.311$, replicated G-test, Fig. 6b). By contrast to those results, TMTT enhanced the attractivity for *P. persimilis* when the LjPT3-derived HIPVs, the active infochemicals, were blended ($G_p = 30.839, P < 0.001$, replicated G-test). The predators showed a strong preference for infested LjPT3 plants (HIPVs + TMTT) over infested WT plants (HIPVs) ($G_p = 30.839, P < 0.001$, replicated G-test).

Moreover, both *N. californicus* and *P. persimilis* females showed only a non-significant tendency to prefer volatiles from uninfested LjPT5 plants emitting low levels of TMTT (Figs. 4b and S7a), when compared to those from uninfested WT plants [*N. californicus*: $G_p = 1.807, P = 0.179$; *P. persimilis*: $G_p = 0, P = 1$, replicated G-test, Fig. S7b and S7c]. The same held in the case of comparison to volatiles from infested LjPT5 plants (HIPVs + low levels of TMTT vs HIPVs) [*N. californicus*: $G_p = 1.253, P = 0.263$; *P. persimilis*: $G_p = 1.807, P = 0.179$, replicated G-test, Fig. S7b and S7c].

**Discussion**

*In vitro* and *in planta* conditions show a different product spectrum of PITPS2

The composition of the induced volatile blends that affects specific plant-arthropod interactions depends on the product spectrum of TPSs (Arimura *et al.*, 2009). *In vitro*, PITPS2 enables the conversion of three prenyl diposphosphate substrates: GDP, FDP and GGDP. PITPS2 belongs to the TPS-g family, in which many homologues convert at least two, and in some cases three, prenyl diposphosphate substrates (Fig. S1). Similar multifunctional, multisubstrate TPS-g enzymes producing terpene alcohols have been
characterized from rice, snapdragon, tomato, grape, and strawberry (reviewed in Tholl et al. (2011)).

In vitro assays with the recombinant PITPS2 enzyme extracted from E. coli showed that the monoterpene linalool is the predominant product, although the sesquiterpene (E)-nerolidol and the diterpene GL are produced at 82% and 16% of the levels of linalool, respectively (Fig. 1). Yet, transgenic L. japonicus plants expressing PITPS2 generated GL and its degradation product TMTT, but neither linalool nor (E)-nerolidol. Therefore, it can be assumed that independent TPS provides linalool and the precursor for DMNT formation in lima bean and L. japonicas. Moreover, differences of the biochemical conditions between plant and microbial expression systems may in many cases cause distinct product spectra, as shown in the catalysis of TPSs, where a divalent metal ion such as Mg$^{2+}$ or Mn$^{2+}$ is required (Köllner et al., 2004). The heterologous expression of geraniol synthase (GES) from Ocimum basilicum in various microbial (Saccharomyces cerevisiae and E. coli) and plant (Vitis vinifera, Arabidopsis, and Nicotiana benthamiana) systems revealed that heterologous expression in various plant and bacterial systems greatly influences the amount of the GES products in leaf tissues or culture media, and the qualitative profile in the metabolically engineered in vivo conditions (Fischer et al., 2012). The functional properties of TPS are, therefore, likely to depend not only on the enzyme’s amino-acidic sequence but also on the cellular compartment, especially when comparing between plant and bacterial systems.

More notably, genetic engineering of TPSs in plants does not always cause production of substantial amounts of terpenes in transgenic plants, most probably...
because of a lack of sufficient precursors in the protein-targeted cellular components (Hohn & Ohlrogge, 1991; Wallaart et al., 2001). This would hold true for both transgenic *L. japonicus* and *N. tabacum* plants expressing MtTPS3 in the current study. Similarly to PlTPS2, MtTPS3 appears to be targeted to the plastids (Gomez et al., 2005), and in vitro assays have shown that MtTPS3 predominantly converts FDP to (E)-nerolidol (Arimura et al., 2008), whereas FDP, the precursor for sesquiterpenes, is not sufficiently available in the plastid (Wu et al., 2006). Alternatively, the failure of de novo synthesis of MtTPS3 proteins and/or their folding is also a possible cause.

Degradation of GL leads to TMTT formation

GL, the PlTPS2 product, appears to be converted to TMTT via an oxidative C-C bond cleavage reaction in plants (Donath & Boland, 1994; Donath & Boland, 1995; Piel et al., 1998), as proven using transgenic *L. japonicus* plants in the current study and transgenic Arabidopsis plants in a previous study (Herde et al., 2008). In Arabidopsis, the herbivore-induced biosynthesis of TMTT appears to be catalyzed by CYP82G1, a P450 of the so-far uncharacterized plant CYP82 family (Lee et al., 2010). Recombinant CYP82G1 has shown narrow substrate specificity for GL and its C(15)-analog (E)-nerolidol, which is converted to the respective DMNT (Lee et al., 2010). Notwithstanding, GL was not successfully converted to TMTT in any of the WT or transgenic *N. tabacum* plants, implying a lack of potential conversion via a CYP82G1 homologue in this species.
Absence of direct defenses against sucking herbivores by GL or TMTT in transgenic plants

Since the direct defensive properties of either GL or TMTT against herbivorous pests had not hitherto been proved, we tested them using our transgenic system. Although LjPT3 lines produced GL and TMTT, they were not detrimental to the growth or survival of offspring of sucking herbivore species *T. urticae* or *L. trifolii* (Figs. 5 and S6). It has also been reported that feeding on the leaves of transgenic *N. tabacum* plants that produced linalool did not affect the larval survival or larval mass of *Helicoverpa armigera* (McCallum *et al.*, 2011). In contrast, *Brevicoryne brassicae* was repelled by these transgenic Arabidopsis lines expressing a linalool/nerolidol synthase gene *FaNES1*, although the performance of this pest was not affected (Kos *et al.*, 2013).

TMTT enhances the attraction of predatory mites in transgenic plants

Two predator species exhibited different olfactory responses to LjPT3 lines. In summary, it was observed that *N. californicus* is attracted to uninfested transgenic plants but not by *T. urticae*-infested transgenic plants, whereas *P. persimilis* is attracted to infested transgenic plants but not to uninfested transgenic plants, in comparison to the attraction by uninfested or infested WT plants. However, the other transgenic plants (LjPT5) emitting low levels of TMTT were preferred neither by *N. californicus* nor *P. persimilis*, when they were uninfested or infested, in comparison to the attraction by uninfested or infested WT plants. Notably, those results imply the following features.
Firstly, some suitable levels of TMTT appear to be an attractant for *N. californicus*. However, when the entire blend of HIPVs is mixed, the predator mites cease to discriminate transgenic plants from WT plants. This is probably because HIPVs in the blend emitted from the infested *L. japonicus* (MG-20) plants [consisting mainly of (Z)-3-hexen-1-yl acetate, (E)-β-ocimene and DMNT (Arimura *et al.*, 2004)] confer a full ability to attract the mites, hiding the attractivity of a TMTT cue. A blend of HIPVs was previously shown to be the most powerful attractant for *N. californicus*, as compared with individual HIPV cues found in the odorant blends from *T. urticae*-infested lima bean leaves and physically damaged leaves [(Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl acetate and (E)-2-hexenal, and linalool], except for the methyl salicylate cue (Shimoda, 2010). It was also shown that methyl salicylate was preferred by *N. californicus* equally to an HIPV blend (Shimoda, 2010). TMTT is, therefore, thought to be a strong attractant for *N. californicus*, but not stronger than a blend of volatiles from *T. urticae*-induced *L. japonicus* plants, and it is unlikely that there is an additive effect when TMTT and the blend are mixed.

Secondly, TMTT appears to act as a supporting infochemical for the attraction of another predator, *P. persimilis*, when added to an active, natural HIPV blend. This trend was very similar to that observed when transgenic torenia plants emitting (E)-β-ocimene were used: the trans-volatile enhanced the ability to attract *P. persimilis* only when added to a HIPV blend from the infested transgenic torenia plants, in comparison to the attraction by infested WT plants (Shimoda *et al.*, 2012). This is partly in line with results from van Wijk *et al.* (2008) showing that 30 individual HIPV compounds,
including TMTT, are no more attractive or repellent for *P. persimilis* than control vapors which are induced in plants fed upon by spider mites, with only three exceptions [octan-1-ol, (Z)-3-hexen-1-ol and methyl salicylate]. These results indicate that an individual HIPV has no a priori meaning to *P. persimilis*.

In conclusion, our data suggest that the attractiveness of TMTT depends on the predatory mite species because of the background odors which synergize with the homoterpene to attract these mites. However, in the case of transgenic torenia plants emitting (E)-β-ocimene, the enhancing effect of the transgenic plant product embedded in endogenous HIPVs was even masked by floral volatiles. Also, in another case, a blend of HIPVs included repellent or inhibitory cues (e.g., oximes) that caused masking of the attractiveness of methyl salicylate to *P. persimilis* (Kappers *et al.*, 2011). Because of such complexity, the use of transgenic plants might substantially contribute to ecological studies aimed to evaluate infochemical-mediated interactions between plants and arthropods in a background of several odors.

In summary, TMTT appears to attract different types of predators of spider mites in different manners (Fig. 7). *P. persimilis* is a voracious, specialized predator of *Tetranychus* mites, whereas *N. californicus* is a generalized feeder that consumes pollen, mites, thrips and other tiny arthropods (McMurtry & Croft, 1997). In other words, *P. persimilis* is probably better adapted to high density than to low density of *T. urticae* prey. Overly rapid predation of *Tetranychus* mites would occasionally result in the lack of prey if the prey density were low (Walzer *et al.*, 2001). LjPT3 lines and *P. persimilis* would both benefit from the fact that the enhanced attraction of *P. persimilis* by HIPVs...
blended with TMTT would assist the predators to search for *T. urticae*-damaged plants only when the prey density was high. In contrast, *N. californicus* can survive even at low densities of *T. urticae* prey by flexibly switching their prey (Walzer *et al.*, 2001). This fact is beneficial to both *N. californicus* and its host LjPT3 lines, because the activity of TMTT, irrespective of the presence of HIPVs, for *N. californicus* would enable the host plants to guard themselves before *T. urticae* invaded or when the prey density was low. Our study therefore suggests that the manipulation of TMTT is an ideal platform for *Tetranychus* mite control by attracting at least two predators via different strategies. However, it remains to be elucidated how low and high density of mites can potentially influence the activity of transgenic plant volatiles (TMTT) for generalist and specialist predators; and whether the current transgenic plant approach can significantly benefit crops by protecting them from *Tetranychus* mites in real agricultural settings.

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Supporting Information

Fig. S1. Sequence relatedness of Lima bean TPS2 (PITPS2; GenBank accession no. KC012520), Medicago truncatula TPS3 (MtTPS3; AAV36466) and some other angiosperm TPSs.

Fig. S2. Relative mRNA levels of PITPS2 or MtTPS3 in leaves of in transgenic plants.

Fig. S3. Morphology of transgenic N. tabacum plants.

Fig. S4. Morphology of transgenic L. japonicus plants.

Fig. S5. Representative gas chromatography-mass spectrometry profile of volatiles emitted from the WT and transgenic N. tabacum (NtPT or NtMT) lines.

Fig. S6. Survival rate of L. trifolii from larva to pupal stages in WT and LjPT3 lines.

Fig. S7. TPS products and olfactory response of the predatory mites to LjPT5 lines.

Table S1. Primers used for this study.
Figure legends

**Fig. 1** Terpenes formed by the extracts containing the recombinant PITPS2 enzymes. The PITPS2 products from assays of the extract prepared from the BL21-CodonPlus(DE3) strain transformed with the recombinant vector (pHis8.3-PITPS2), with GDP (a), FDP (b) or GGDP (c) as substrate, are illustrated. IS, internal standard. (d) Absolute values for the PITPS2 product from assays (ng/µg protein/h). Data represent the mean ± SE (n = 4).

**Fig. 2** Effect of treatment of jasmonic acid (JA), infestation with spider mites, and alamethicin (ALA) on the expression level of PITPS2 (a) and biosynthesis of linalool, DMNT, TMTT and GL (b) in lima bean leaves. Treatments: JA (0.5 mM), ALA (0.1 mM) or spider mites (40 females per plant). Data represent the mean ± SE (a, n = 3-4; b, n = 4-7). An asterisk (*) indicates that treated plants were significantly different from untreated plants (0 h) (P < 0.05, Dunnett’s test). (c) Schematic representation of pGWB452 (35S promoter-G3GFP) and pGWB451-PITPS2 (35S promoter-PITPS2-G3GFP) and pGWB451-PITPS2. Gateway, the Gateway cassette; P35S, 35S promoter; Tnos, nopaline synthase terminator. (d) Subcellular localization of PITPS2-GFP fusion protein in lima beans. Leaves were transformed with the control plasmid pGWB452 or pGWB451-PITPS2 by agroinfiltration. Images were taken with a confocal laser scanning microscope. GFP, GFP fluorescence image false-colored in green; chlorophyll, chlorophyll autofluorescence image false-colored in red; Merged, merged of chlorophyll and GFP fluorescence images. Bars = 200 µm.
Fig. 3 GL accumulated in WT and transgenic *N. tabacum* lines. Values represent the means + SE (n = 3-4). An asterisk (*) indicates that transgenic lines were significantly different from WT (*P* < 0.05, Dunnett’s test).

Fig. 4 Formation of TPS products in transgenic *L. japonicus* lines. (a) Representative gas chromatography-mass spectrometry profiles of volatiles emitted from the WT and transgenic lines not infested or infested with *T. urticae* for 2 days. 1. (Z)-3-hexen-1-yl acetate; 2. (E)-β-ocimene; 3. DMNT; 4. TMTT; IS, internal standard. IS, internal standard. Values for DMNT, TMTT and GL formation are shown in b. Values represent the means + SE (n = 3-5). An asterisk (*) indicates that a transgenic line was significantly different from WT (*P* < 0.05, Dunnett’s test).

Fig. 5 Effect of transgenic plants on herbivore performance. (a) Survival rate of adult female *T. urticae* and (b) total number of their eggs (means + SE) on the leaves of WT, GUS, LjPT3 and LjMT6 lines 3 days after inoculation. ns (survival rate: *P* > 0.05, *df* = 2, GLM-test; oviposition: *P* > 0.05, *df* = 2, ANOVA). (c) Survival rate of *T. urticae* offspring (from larva to adult stages) on the leaves of WT, GUS, LjPT3 and LjMT6 lines. ns (ns, *P* > 0.05, *df* = 2, GLM-test).

Fig. 6 Olfactory response of *N. californicus* (a) or *P. persimilis* (b) when offered infested WT plants, uninfested LjPT3 or infested LjPT3 plants vs uninfested or infested
WT plants. The figures in parentheses represent the numbers of predators that did not choose either odor source (‘no choice’ subjects). A replicated G-test was conducted to evaluate the significance of attraction in each experiment (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.005$; ns, $P > 0.005$).

Fig. 7 Schematic presentation of effect of transgenic LjPT lines on the attraction of predatory mites.
Fig. 1

253x190mm (300 x 300 DPI)
Fig. 2

253x190mm (300 x 300 DPI)
Fig. 3

253x190mm (300 x 300 DPI)
Fig. 4

253x190mm (300 x 300 DPI)
Fig. 5

253x190mm (300 x 300 DPI)
Fig. 6

253x190mm (300 x 300 DPI)
Fig. 7

Transgenic *L. japonicus* plants

Emitted constitutively

Emitted inducibly

*T. urticae* attack

Relative predator attraction

**EQUAL** *P. persimilis*

**HIGH** *N. californicus*  
(vs uninfested WT)

**HIGH** *P. persimilis*  
(vs infested WT)

**EQUAL** *N. californicus*  
(vs infested WT)

253x190mm (300 x 300 DPI)