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Metabolic engineering of the C_{16} homoterpene TMTT in *Lotus japonicus* through overexpression of (*E*,*E*)-geranyllinalool synthase attracts generalist and specialist predators in different manners

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Summary

• Plant defenses against herbivores include the emission of specific blends of volatiles, which enable plants to attract natural enemies of herbivores.

• We characterized a plastidial terpene synthase gene, *PlTPS2*, from lima bean. The recombinant PlTPS2 protein was multi-functional, producing linalool, (*E*)-nerolidol and (*E*,*E*)-geranyllinalool, precursors of (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [TMTT].

• Transgenic *Lotus japonicus* and *Nicotiana tabacum* plants, expressing *PlTPS2* or its homologue *Medicago truncatula TPS3* (*MtTPS3*), were produced and used for bioassays with herbivorous and predatory mites. Transgenic *L. japonicus* plants expressing PlTPS2 produced (*E*,*E*)-geranyllinalool and TMTT, whereas wild-type plants and transgenic plants expressing MtTPS3 did not. Transgenic *N. tabacum* expressing PlTPS2 produced (*E*,*E*)-geranyllinalool but not TMTT. Moreover, in olfactory assays, the generalist predatory mite *Neoseiulus californicus* but not the specialist *Phytoseiulus persimilis* was attracted to uninfested, transgenic *L. japonicus* plants expressing PlTPS2 over wild-type plants. The specialist *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.

Introduction

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_{10}), sesquiterpenes (C_{15}), and tetranor-terpenoids (homoterpenes, (*E*)-4,8-dimethyl-1,3,7-nonatriene [DMNT, C_{11}] or (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [TMTT, C_{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 PlTPS2 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 puerariicola* 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.

Chemical and herbivore treatment

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)$_{12\text{-}18}$ 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 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 diphoshate (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™-5 capillary column (0.25 mm i.d.

184 x 15 m with 0.25-mm film, Alltech, Deerfield, IL, USA). Injection volume: 1 µl; split
185 1:100; 220°C. Ionization energy: 70 eV. Compounds were eluted under programmed
186 conditions starting from 40˚C (2-min hold) and ramped up at 10˚C min^{-1} to 200˚C
187 followed by 30˚C min^{-1} to 280˚C, which was held for 1 min prior to cooling. Helium at
188 a flow rate of 1.5 ml min^{-1} served as a carrier gas. The products were identified and
189 quantified as described previously (Arimura *et al.*, 2008).
190
191 Generation of transgenic *L. japonicus* and *N. tabacum* plants
192 The full-length coding region of lima bean *PlTPS2* (GenBank accession no. KC012520)
193 or *MtTPS3* (AY766249) was inserted into binary vector pMDC32 using the Gateway
194 cloning system (Invitrogen). The resulting plasmid, pMDC32-*PlTPS2* or
195 pMDC32-*MtTPS3*, was transformed into *Agrobacterium tumefaciens* strain EHA105 by
196 electroporation. Tobacco plants that had been aseptically grown from seeds for about 1
197 month were transformed via an *A. tumefaciens*-mediated leaf disc procedure (Horsch *et*
198 *al.*, 1985). *L. japonicus* was also transformed using the *A. tumefaciens*-mediated
199 transformation procedure described by Imaizumi *et al.* (2005). As the selection agent,
200 30 mg l^{-1} or 50 mg l^{-1} hygromycin was used for *N. tabacum* or *L. japonicus*,
201 respectively. After rooting and acclimatization, the regenerated plants were grown in a
202 closed greenhouse to set seeds. About 8 lines of transgenic T_1 seeds from each
203 transformant were tested for germination on 1/2 Murashige and Skoog medium
204 supplemented with 20-30 mg l^{-1} hygromycin. T_2 seeds harvested from each individual
205 T_1 plant that showed ca. 3:1 segregation ratio were tested for hygromycin-resistance

206 again. Both T_1 and T_2 plant lines were used for further chemical and gene expression
207 analyses, and homozygous T_3 plant lines were used in bioassays. A homozygous *L.*
208 *japonicas* line transformed with the binary plasmid pIG121Hm, expressing hygromycin
209 phosphotransferase (*hpt*) and intron-containing β-glucuronidase [GUS] genes (Hiei *et*
210 *al.*, 1994), was used as a control.
211
212 Quantitative reverse transcription (RT)-PCR
213 Total RNA was isolated from leaf tissues using a Qiagen RNeasy Plant Mini Kit and an
214 RNase-Free DNase Set (Qiagen) following the manufacturer's protocol. First-strand
215 cDNA was synthesized using a PrimeScript RT reagent Kit (Takara, Otsu, Japan), and
216 0.5 µg of total RNA at 37˚C for 15 min. Real-time PCR was performed on an ABI
217 Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA)
218 using FastStart Universal SYBR Green Master (ROX) (Roche Applied Science,
219 Indianapolis, IN, USA), cDNA (1 µl from 10 µl of each RT product pool), and 300 nM
220 primers. The following protocol was used: initial polymerase activation: 10 min at
221 95°C; then 40 cycles of 15 s at 95°C and 60 s at 60°C. PCR conditions were chosen by
222 comparing threshold values in a dilution series of the RT product, followed by a
223 non-RT template control and non-template control for each primer pair. Relative RNA
224 levels were calibrated and normalized with the level of *PlACT1* mRNA (GenBank
225 accession no. DQ159907), *LjTUB* (AB510590) or *NtACT* (GQ281246). Primers used
226 for this study are shown in Table S1.
227

Transient expression of green fluorescent protein (GFP) fusion proteins

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 $MgCl_2$ and 150 mM acetosyringone (Sigma-Aldrich). The bacterial suspensions were adjusted to OD_{600} 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).

Volatile analysis

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, CO_2 <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 (7HG-G010-11, 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

293 control following the above procedure.

294

295 Assay for herbivores

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

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

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

313

314 Olfactory assay

315 Each olfactory bioassay was performed using a Y-tube olfactometer in a laboratory
316 (25°C, light intensity of 80 μE m^{-2} s^{-1}), according to the method described by Shimoda
317 *et al.* (2012). The odor sources used were divided into the following six types:
318 uninfested WT plants, infested WT plants, uninfested LjPT3 plants, infested LjPT3
319 plants, uninfested LjPT5 plants, and infested LjPT5 plants. For infestation, a potted
320 plant in a plastic pot was infested with *T. urticae* (50 adult females) for 2 days. For each
321 assay, 10 intact plants (each plant weighing about 2.5 g ; 5 plants / pot) were used as an
322 odor source.

323 Predators were individually introduced at the start point in the olfactometer, and
324 the numbers of predators choosing either sample or control odor sources were recorded.
325 Predators that did not choose within 5 min ('no choice' subjects) were excluded from
326 the statistical analysis. Assays using 20 predators were carried out as a single replicate
327 in a day. Four replications (i.e. 80 predators in all) were carried out on different days.
328 The results from three or four replications of each experiment were subjected to a
329 replicated *G*-test; the pooled *G*-value (*G*p, df = 1 in each) was used to test the null
330 hypothesis that the predators exhibited a 50 : 50 distribution over the sample and control
331 odor sources in each experiment (Sokal & Rohlf, 1995). We also confirmed that there
332 was no significant heterogeneity among replications in each experiment (df = 3, $P >$
333 0.05 for each *G*h, replicated *G*-test), suggesting good reproducibility of the two-choice
334 test.
335
336 **Results**

Functional characterization of PlTPS2

In order to verify the functional involvement of *PlTPS2* 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 *PlTPS2* encodes a predicted protein of 569 amino acids that shares 78% identity and 83% similarity with a *Glycine max* predicted (3S,6*E*)-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 PlTPS2 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 *PlTPS2* cDNA insert did not produce any terpene products (data not shown).

Expression of PlTPS2, 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 *PlTPS2* upon external application of jasmonic acid (JA, 0.5

359 mM), *T. urticae* spider mite feeding, or alamethicin (ALA, 0.1 mM), an elicitor of the
360 plant pathogenic fungus *Trichoderma viride* (Arimura *et al.*, 2008) (Fig. 2a). ALA
361 induced *PlTPS2* transcripts at 2 h, and more dramatically at 6 and 24 h, after application.
362 Similarly, *PlTPS2* transcript levels were increased gradually over the time-course of
363 exposure of lima bean leaves to spider mites (~24 h). In contrast to these stimuli,
364 however, JA application did not induce the transcript, indicating the lack of dependence
365 of *PlTPS2* activation on JA signaling (Fig. 2a).

366 Next, to test if the transcriptional profile of *PlTPS2* was reflected by the emission
367 of linalool and of the homoterpenes DMNT and TMTT, we measured headspace
368 volatiles emitted from lima bean plants exposed to spider mite feeding and from leaves
369 treated with JA or ALA (Fig. 2b). The oxidative degradation of (*E*)-nerolidol and GL
370 generates DMNT and TMTT (Donath & Boland, 1994), and these volatiles have been
371 found in the blend of HIPVs from lima beans exposed to ALA or spider mites (Ozawa *et*
372 *al.*, 2000; Engelberth *et al.*, 2001). As expected, emission of DMNT and TMTT from
373 lima bean plants was induced, in similar manner to the *PlTPS2* transcriptional profile, by
374 ALA treatment or spider mite feeding. Emission of these homoterpenes, however,
375 remained undetectable in JA-treated plants, in contrast to the emission of linalool,
376 which was elevated only when JA was provided. GL, one of the PlTPS2 products, was
377 hardly detected in the headspace of any of the lima bean samples, whereas this diterpene
378 accumulated in leaves infested with spider mites or treated with ALA (Fig. 2b).
379
380 Subcellular localization of PlTPS2

381 The subcellular localization of PlTPS2-GFP fusion proteins (Fig. 2c) in transiently
382 expressing lima bean leaf cells was plastidial (Fig. 2d). This result reflected the
383 plastidial localization of MtTPS3, a homologue of PlTPS2 (Gomez *et al.*, 2005) but not
384 the localization of Arabidopsis GL synthase (AtGES), which is targeted to the cytosol or
385 the endoplasmic reticulum (Herde *et al.*, 2008).
386
387 Transgenic plants expressing *TPSs*
388 In order to understand the physiological and ecological features of *PlTPS2* and its *M.*
389 *truncatula* homologue *MtTPS3* (Arimura *et al.*, 2008), transgenic plants constitutively
390 expressing these genes were generated. The respective ORF sequences under the control
391 of the CaMV 35S promoter were transformed into *N. tabacum* and *L. japonicus*,
392 resulting in four individual lines (NtPT [*N. tabacum* expressing *PlTPS2*], NtMT [*N.*
393 *tabacum* expressing *MtTPS3*], LjPT [*L. japonicus* expressing *PlTPS2*], and LjMT [*L.*
394 *japonicus* expressing *MtTPS3*]). Following selection for hygromycin resistance in the T_1
395 and T_2 plant lines, positive plants were grown and used for further experiments. All the
396 transgenic lines exhibited trans-gene (*PlTPS2* or *MtTPS3*) expression in the leaves,
397 whereas wild-type (WT) plants did not (Fig. S2). In addition, none of the transgenic
398 lines exhibited any detectable differences in their morphology (Figs. S3 and S4).
399 None of the transgenic tobacco lines used for analysis exhibited detectable levels
400 or increased emission of the TPS-derived volatiles (i.e., linalool, (*E*)-nerolidol, DMNT
401 and TMTT; Fig. S5). The non-volatile diterpene GL was, however, accumulated at
402 significantly higher levels in the leaves of NtPT lines than in the WT (P<0.05, Dunnett's

403 test) (Fig. 3). This diterpene was very slightly elevated in NtMT lines when compared to
404 its level in WT (NtMT3, 1.9 times; NtMT4, 1.4 times, $P > 0.05$, Dunnett's test).
405 Interestingly, substantial production of both TMTT and GL was detected only in the
406 leaves of transgenic *L. japonicus* plants expressing *PlTPS2* (LjPT3 and 5; $P < 0.05$,
407 Dunnett's test), whereas WT, GUS-transgenic control plants, and transgenic plants
408 expressing *MtTPS3* (LjMT3 and 6) showed no detectable production (Fig. 4). In
409 response to spider mite attack, both LjPT3 and LjPT5 plants emitted TMTT, at similar
410 levels as the uninfested plants (Fig. 4b). In contrast, after infestation, the accumulation
411 of GL decreased to 44% and 32% of that in uninfested LjPT3 and LjPT5 plants,
412 respectively. The emission of TMTT was not observed in uninfested WT and LjMT6
413 plants and very slightly increased in response to spider mite attack, although GL was
414 observed neither in uninfested nor in infested plants. DMNT was similarly emitted from
415 infested WT, LjPT3 and LjMT6 plants (Fig. 4b).

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

420

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

422 We evaluated the effects of transgenic plant products on survival, oviposition and
423 development of pest herbivores. *T. urticae* females survived and reproduced
424 equivalently among WT, GUS, LjPT3, and LjMT6 lines (survival: $\chi^2 = 2.888$, $df = 3$, P

425 = 0.409, GLM-test, Fig. 5a; oviposition: F = 1.477, df = 3, P = 0. 223, ANOVA, Fig.
426 5b). Moreover, no significant difference was observed among those plants in the
427 development of the next generation of *T. urticae* (survival ratio from larva to adult: χ^2 =
428 1.027, df = 3, P = 0.795, GLM-test, Fig. 5c). We evaluated another herbivore species,
429 the American serpentine leafminer *L. trifolii*, and found that its larvae developed
430 similarly between WT and LjPT3 (survival ratio from larval to pupal stages: χ^2 = 3.127,
431 df = 1, P = 0.077, GLM-test, Fig. S6).
432
433 Olfactory response of transgenic plants
434 We assessed the influence of HIPVs and the trans-volatile TMTT on the olfactory
435 responses of *N. californicus* females. *N. californicus* is a generalist feeder that can
436 exploit various foods such as small insects and pollen, as well as species of the genus
437 *Tetranychus* (Shimoda, 2010). The predators showed significant preferences for HIPVs
438 from *T. urticae*-infested WT plants of *L. japonicus* (Gp= 4.312, P < 0.05, replicated
439 G-test, Fig. 6a) and TMTT from uninfested LjPT3 plants (Gp= 7.312, P < 0.01,
440 replicated G-test), in comparison to basal volatiles from uninfested WT plants. However,
441 the predators did not discriminate between infested LjPT3 (HIPVs + TMTT, see Fig. 4)
442 and infested WT plants (HIPVs) (Gp= 0.450, P = 0.502, replicated G-test), indicating
443 that TMTT had no additive effect on the attractivity of HIPVs for *N. californicus*.
444 We next assessed olfactory responses of *P. persimilis* females. *P. persimilis* is a
445 specialist predator that needs abundant *Tetranychus* spider mites as prey (Walzer *et al.*,
446 2001; Shimoda *et al.*, 2012). The predators did not discriminate TMTT from uninfested

447 LjPT3 plants from basal volatiles from uninfested WT plants (*G*p= 1.028, *P* = 0.311,
448 replicated G-test, Fig. 6b). By contrast ~~to those results~~, TMTT enhanced the attractivity
449 for *P. persimilis* when the LjPT3-derived HIPVs, the active infochemicals, were
450 blended (*G*p= 30.839, *P* < 0.001, replicated G-test). The predators showed a strong
451 preference for infested LjPT3 plants (HIPVs + TMTT) over infested WT plants (HIPVs)
452 (*G*p= 30.839, *P* < 0.001, replicated G-test).

453 Moreover, both *N. californicus* and *P. persimilis* females showed only a
454 non-significant tendency to prefer volatiles from uninfested LjPT5 plants emitting low
455 levels of TMTT (Figs. 4b and S7a), when compared to those from uninfested WT plants
456 [*N. californicus*: *G*p= 1.807, *P* = 0.179; *P. persimilis*: *G*p= 0, *P* = 1, replicated G-test,
457 Fig. S7b and S7c]. The same held in the case of comparison to volatiles from infested
458 LjPT5 plants (HIPVs + low levels of TMTT vs HIPVs) (*N. californicus*: *G*p= 1.253, *P*
459 = 0.263; *P. persimilis*: *G*p= 1.807, *P* = 0.179, replicated G-test, Fig. S7b and S7c).
460

461 **Discussion**

462 *In vitro* and *in planta* conditions show a different product spectrum of PlTPS2

463 The composition of the induced volatile blends that affects specific plant-arthropod
464 interactions depends on the product spectrum of TPSs (Arimura *et al.*, 2009). *In vitro*,
465 PlTPS2 enables the conversion of three prenyl diphosphate substrates: GDP, FDP and
466 GGDP. PlTPS2 belongs to the TPS-g family, in which many homologues convert at
467 least two, and in some cases three, prenyl diphosphate substrates (Fig. S1). Similar
468 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 PlTPS2 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 *PlTPS2* 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.

512

513 Absence of direct defenses against sucking herbivores by GL or TMTT in transgenic
514 plants

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

524

525 TMTT enhances the attraction of predatory mites in transgenic plants

526 Two predator species exhibited different olfactory responses to LjPT3 lines. In
527 summary, it was observed that *N. californicus* is attracted to uninfested transgenic
528 plants but not by *T. urticae*-infested transgenic plants, whereas *P. persimilis* is attracted
529 to infested transgenic plants but not to uninfested transgenic plants, in comparison to the
530 attraction by uninfested or infested WT plants. However, the other transgenic plants
531 (LjPT5) emitting low levels of TMTT were preferred neither by *N. californicus* nor *P.*
532 *persimilis*, when they were uninfested or infested, in comparison to the attraction by
533 uninfested or infested WT plants. Notably, those results imply the following features.

534 Firstly, some suitable levels of TMTT appear to be an attractant for *N.*
535 *californicus*. However, when the entire blend of HIPVs is mixed, the predator mites
536 cease to discriminate transgenic plants from WT plants. This is probably because HIPVs
537 in the blend emitted from the infested *L. japonicus* (MG-20) plants [consisting mainly
538 of (*Z*)-3-hexen-1-yl acetate, (*E*)-β-ocimene and DMNT (Arimura *et al.*, 2004)] confer a
539 full ability to attract the mites, hiding the attractivity of a TMTT cue. A blend of HIPVs
540 was previously shown to be the most powerful attractant for *N. californicus*, as
541 compared with individual HIPV cues found in the odorant blends from *T.*
542 *urticae*-infested lima bean leaves and physically damaged leaves [(*Z*)-3-hexen-1-ol,
543 (*Z*)-3-hexen-1-yl acetate and (*E*)-2-hexenal, and linalool], except for the methyl
544 salicylate cue (Shimoda, 2010). It was also shown that methyl salicylate was preferred
545 by *N. californicus* equally to an HIPV blend (Shimoda, 2010). TMTT is, therefore,
546 thought to be a strong attractant for *N. californicus,* but not stronger than a blend of
547 volatiles from *T. urticae*-induced *L. japonicus* plants, and it is unlikely that there is an
548 additive effect when TMTT and the blend are mixed.

549 Secondly, TMTT appears to act as a supporting infochemical for the attraction of
550 another predator, *P. persimilis*, when added to an active, natural HIPV blend. This trend
551 was very similar to that observed when transgenic torenia plants emitting (*E*)-β-ocimene
552 were used: the trans-volatile enhanced the ability to attract *P. persimilis* only when
553 added to a HIPV blend from the infested transgenic torenia plants, in comparison to the
554 attraction by infested WT plants (Shimoda *et al.*, 2012). This is partly in line with
555 results from van Wijk *et al.* (2008) showing that 30 individual HIPV compounds,

556 including TMTT, are no more attractive or repellent for *P. persimilis* than control vapors
557 which are induced in plants fed upon by spider mites, with only three exceptions
558 [octan-1-ol, (*Z*)-3-hexen-1-ol and methyl salicylate]. These results indicate that an
559 individual HIPV has no a priori meaning to *P. persimilis*.

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

570 In summary, TMTT appears to attract different types of predators of spider mites
571 in different manners (Fig. 7). *P. persimilis* is a voracious, specialized predator of
572 *Tetranychus* mites, whereas *N. californicus* is a generalized feeder that consumes pollen,
573 mites, thrips and other tiny arthropods (McMurtry & Croft, 1997). In other words, *P.*
574 *persimilis* is probably better adapted to high density than to low density of *T. urticae*
575 prey. Overly rapid predation of *Tetranychus* mites would occasionally result in the lack
576 of prey if the prey density were low (Walzer *et al.*, 2001). LjPT3 lines and *P. persimilis*
577 would both benefit from the fact that the enhanced attraction of *P. persimilis* by HIPVs

578 blended with TMTT would assist the predators to search for *T. urticae*-damaged plants
579 only when the prey density was high. In contrast, *N. californicus* can survive even at
580 low densities of *T. urticae* prey by flexibly switching their prey (Walzer *et al.*, 2001).
581 This fact is beneficial to both *N. californicus* and its host LjPT3 lines, because the
582 attractivity of TMTT, irrespective of the presence of HIPVs, for *N. californicus* would
583 enable the host plants to guard themselves before *T. urticae* invaded or when the prey
584 density was low. Our study therefore suggests that the manipulation of TMTT is an
585 ideal platform for *Tetranychus* mite control by attracting at least two predators via
586 different strategies. However, it remains to be elucidated how low and high density of
587 mites can potentially influence the attractivity of transgenic plant volatiles (TMTT) for
588 generalist and specialist predators; and whether the current transgenic plant approach can
589 significantly benefit crops by protecting them from *Tetranychus* mites in real agricultural
590 settings.
591

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Supporting Information

Fig. S1. Sequence relatedness of Lima bean TPS2 (*PlTPS2*; GenBank accession no. KC012520), *Medicago truncatula* TPS3 (MtTPS3; AAV36466) and some other angiosperm TPSs.

Fig. S2. Relative mRNA levels of *PlTPS2* 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.

754 **Figure legends**

755 **Fig. 1** Terpenes formed by the extracts containing the recombinant PlTPS2 enzymes.
756 The PlTPS2 products from assays of the extract prepared from the
757 BL21-CodonPlus(DE3) strain transformed with the recombinant vector
758 (pHis8.3-*PlTPS2*), with GDP (a), FDP (b) or GGDP (c) as substrate, are illustrated. IS,
759 internal standard. (d) Absolute values for the PlTPS2 product from assays (ng/μg
760 protein/h). Data represent the mean + SE (n = 4).

761

762 **Fig. 2** Effect of treatment of jasmonic acid (JA), infestation with spider mites, and
763 alamethicin (ALA) on the expression level of *PlTPS2* (a) and biosynthesis of linalool,
764 DMNT, TMTT and GL (b) in lima bean leaves. Treatments: JA (0.5 mM), ALA (0.1 mM)
765 or spider mites (40 females per plant). Data represent the mean + SE (a, n = 3-4; b, n =
766 4-7). An asterisk (*) indicates that treated plants were significantly different from
767 untreated plants (0 h) ($P < 0.05$, Dunnett's test). (c) Schematic representation of
768 pGWB452 (35S promoter-*G3GFP*) and pGWB451-*PlTPS2* (35S
769 promoter-*PlTPS2*-*G3GFP*) and pGWB451-*PlTPS2*. *Gateway*, the Gateway cassette;
770 *P35S*, 35S promoter; *Tnos*, nopaline synthase terminator. (d) Subcellular localization of
771 PlTPS2-GFP fusion protein in lima beans. Leaves were transformed with the control
772 plasmid pGWB452 or pGWB451-*PlTPS2* by agroinfiltration. Images were taken with a
773 confocal laser scanning microscope. GFP, GFP fluorescence image false-colored in
774 green; chlorophyll, chlorophyll autofluorescence image false-colored in red; Merged,
775 merged of chlorophyll and GFP fluorescence images. Bars = 200 μm.

776

777 **Fig. 3** GL accumulated in WT and transgenic *N. tabacum* lines. Values represent the
778 means + SE (n = 3-4). An asterisk (*) indicates that transgenic lines were significantly
779 different from WT ($P < 0.05$, Dunnett's test).

780

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

788

789 **Fig. 5** Effect of transgenic plants on herbivore performance. (a) Survival rate of adult
790 female *T. urticae* and (b) total number of their eggs (means + SE) on the leaves of WT,
791 GUS, LjPT3 and LjMT6 lines 3 days after inoculation. ns (survival rate: $P > 0.05$, df =
792 2, GLM-test; oviposition: $P > 0.05$, df = 2, ANOVA). (c) Survival rate of *T. urticae*
793 offspring (from larva to adult stages) on the leaves of WT, GUS, LjPT3 and LjMT6
794 lines. ns (ns, $P > 0.05$, df = 2, GLM-test).

795

796 **Fig. 6** Olfactory response of *N. californicus* (a) or *P. persimilis* (b) when offered
797 infested WT plants, uninfested LjPT3 or infested LjPT3 plants vs uninfested or infested

798 WT plants. The figures in parentheses represent the numbers of predators that did not
799 choose either odor source ('no choice' subjects). A replicated G-test was conducted to
800 evaluate the significance of attraction in each experiment (***, $P< 0.001$; **, $P< 0.01$; *,
801 $P< 0.005$; ns, $P> 0.005$).
802
803 **Fig. 7** Schematic presentation of effect of transgenic LjPT lines on the attraction of
804 predatory mites.

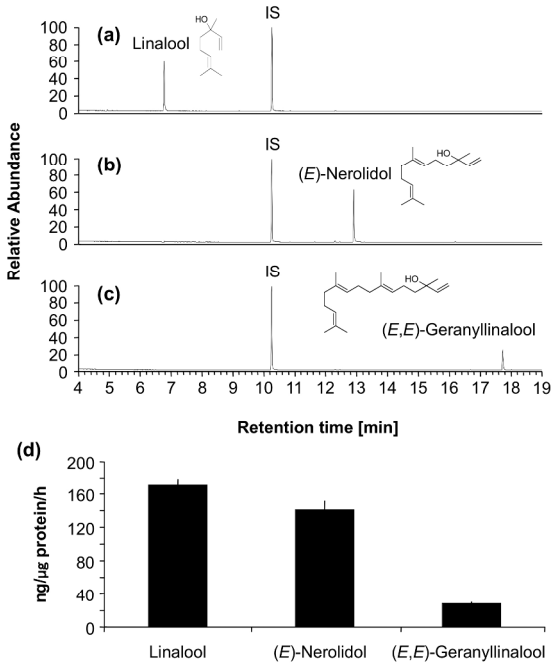

Fig. 1

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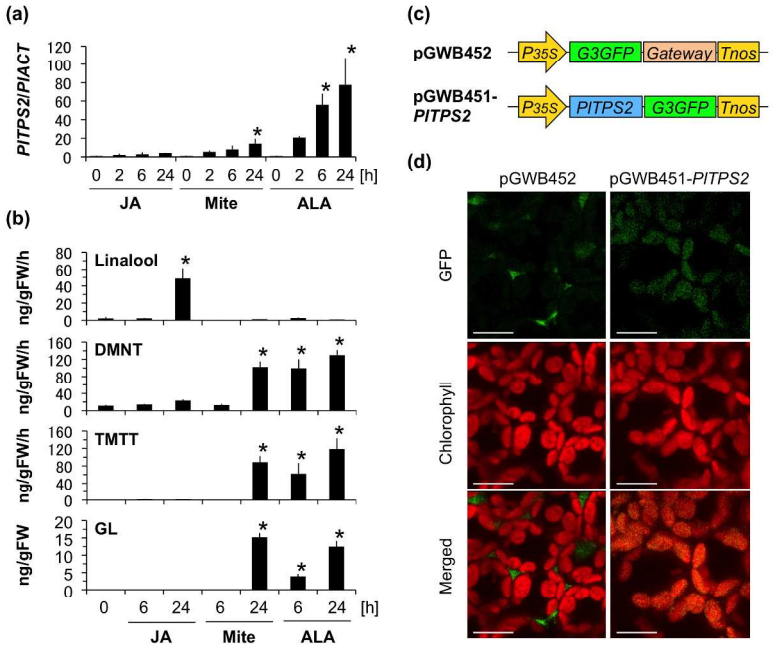

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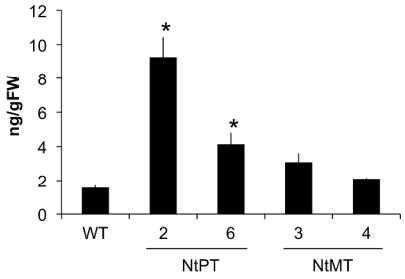

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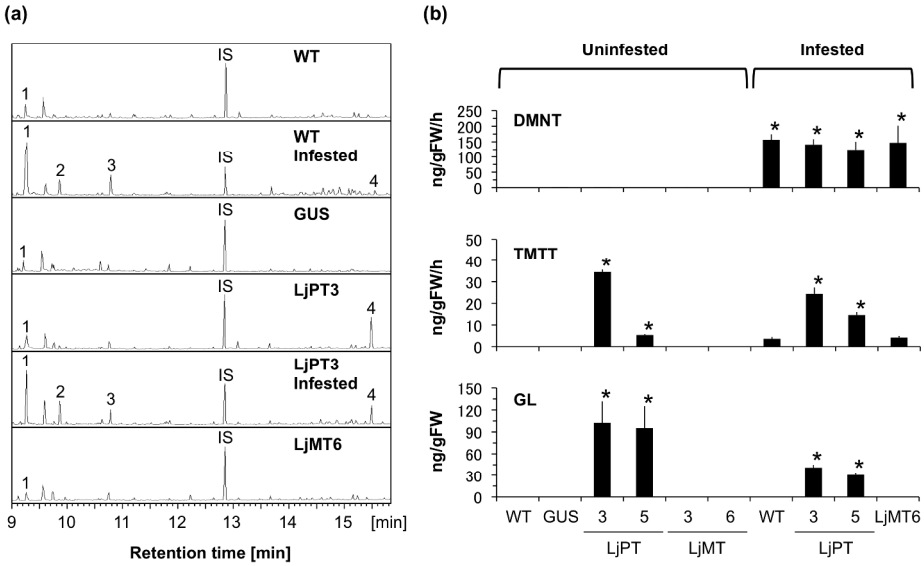

Fig. 4

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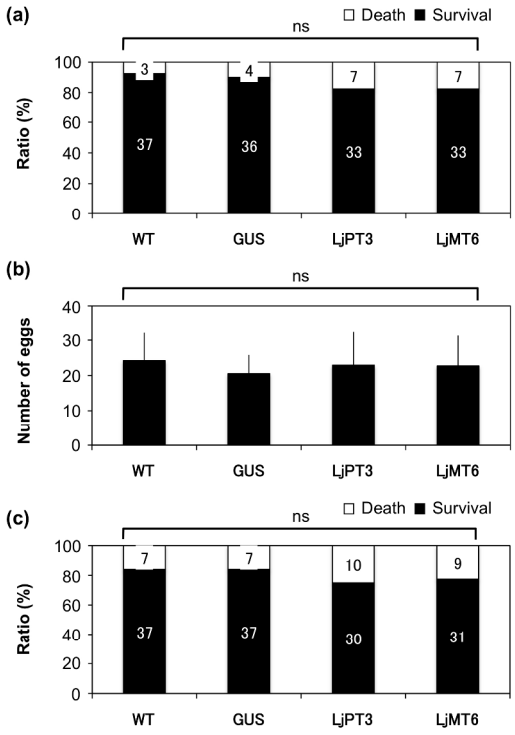

Fig. 5

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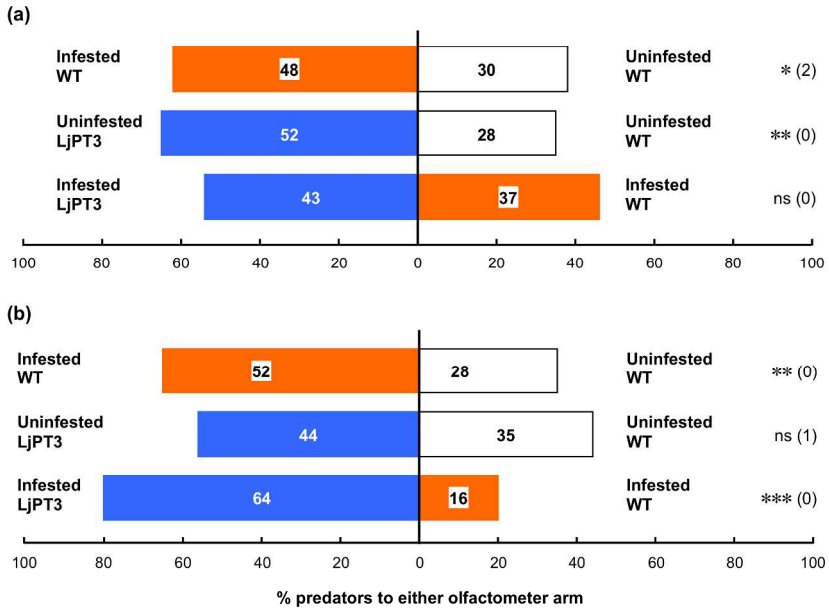

Fig. 6

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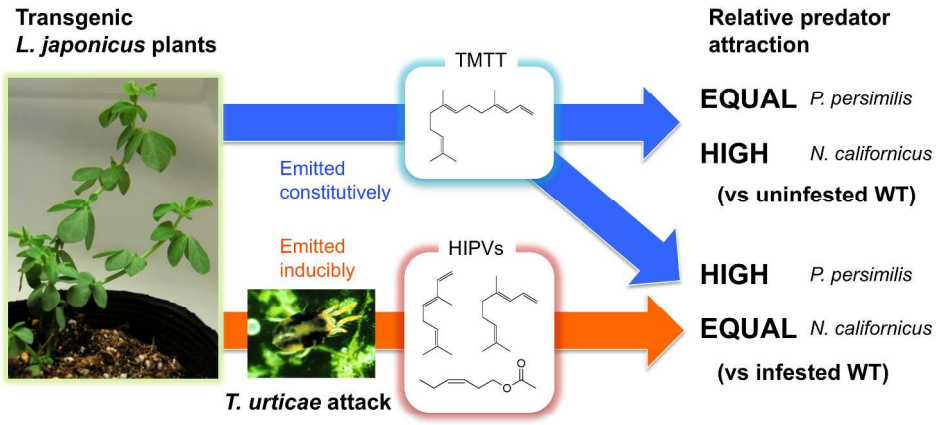

Fig. 7

253x190mm (300 x 300 DPI)