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

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

4
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27 Keywords: herbivore-induced plant volatiles (HIPVs), indirect defense, lima bean, mite,

28 terpene, (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT)

29

For Peer Review

30 **Summary**

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

33 • We characterized a plastidial terpene synthase gene, *PITPS2*, from lima bean. The
34 recombinant PITPS2 protein was multi-functional, producing linalool, (*E*)-nerolidol and
35 (*E,E*)-geranyllinalool, precursors of (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
36 [TMTT].

37 • Transgenic *Lotus japonicus* and *Nicotiana tabacum* plants, expressing *PITPS2* or its
38 homologue *Medicago truncatula* *TPS3* (*MtTPS3*), were produced and used for
39 bioassays with herbivorous and predatory mites. Transgenic *L. japonicus* plants
40 expressing *PITPS2* produced (*E,E*)-geranyllinalool and TMTT, whereas wild-type
41 plants and transgenic plants expressing *MtTPS3* did not. Transgenic *N. tabacum*
42 expressing *PITPS2* produced (*E,E*)-geranyllinalool but not TMTT. Moreover, in
43 olfactory assays, the generalist predatory mite *Neoseiulus californicus* but not the
44 specialist *Phytoseiulus persimilis* was attracted to uninfested, transgenic *L. japonicus*
45 plants expressing *PITPS2* over wild-type plants. The specialist *P. persimilis* was more
46 strongly attracted by the transgenic plants infested with spider mites than by infested
47 wild-type plants.

48 • Predator responses to transgenic plant volatile TMTT depend on various background
49 volatiles endogenously produced by the transgenic plants. Therefore, the manipulation
50 of TMTT is an ideal platform for pest control via the attraction of generalist and
51 specialist predators in different manners.

52 Introduction

53 Indirect defenses of plants against herbivores include the emission of specific blends of
54 volatiles in response to herbivory (HIPVs, herbivore-induced plant volatiles), which
55 enables the plants to attract carnivorous natural enemies of herbivores (Arimura *et al.*,
56 2009; Maffei *et al.*, 2011). Volatile terpenoids are the major products among HIPVs, and
57 in legumes include monoterpenes (C₁₀), sesquiterpenes (C₁₅), and tetranor-terpenoids
58 (homoterpenes, (*E*)-4,8-dimethyl-1,3,7-nonatriene [DMNT, C₁₁] or
59 (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [TMTT, C₁₆]) (Ozawa *et al.*, 2000;
60 Leitner *et al.*, 2005). The molecular diversity of terpenes is expanded as a result of the
61 use by terpene synthases (TPSs) of different prenyl diphosphates as substrates, with
62 these prenyl diphosphates being derived from the mevalonate (MVA) pathway in the
63 cytosol/endoplasmic reticulum or the 2-C-methyl-D-erythritol 4-phosphate (MEP)
64 pathway in plastids (Lange *et al.*, 2000). TPSs are often multi-product enzymes, and
65 thus even a single TPS can contribute significantly to the plasticity of blends, especially
66 blends produced in response to herbivory (Köllner *et al.*, 2004).

67 In addition, during the last decade several other types of multi-functional TPSs
68 have been studied, e.g., in *Medicago truncatula*, MtTPS3, which encodes a
69 multi-functional enzyme producing linalool, (*E*)-nerolidol (precursor of DMNT) and
70 (*E,E*)-geranylinalool (GL, precursor of TMTT) from different prenyl diphosphates
71 serving as substrates (Arimura *et al.*, 2008). In turn, it has been shown that the
72 herbivore-induced biosynthesis of TMTT is catalyzed by the concerted activities of
73 AtGES, a monofunctional enzyme producing GL (Herde *et al.*, 2008), and CYP82G1

74 (Lee *et al.*, 2010) in *Arabidopsis* (*Arabidopsis thaliana*). Of interest is the fact that
75 AtGES is not localized to the plastids, where diterpene synthases are primarily located,
76 but rather resides in the cytosol or in the endoplasmic reticulum. It is likely that the
77 AtGES substrate geranylgeranyl diphosphate (GGDP) is present in these compartments,
78 since there are two *Arabidopsis* GGDP synthases with a localization pattern similar to
79 that observed for AtGES (Okada *et al.*, 2000). In turn, CYP82G1, a cytochrome P450
80 monooxygenase of the *Arabidopsis* CYP82 family, is responsible for the breakdown of
81 GL to the insect-induced TMTT. Homology-based modeling and substrate docking
82 support an oxidative bond cleavage of the alcohol substrate via syn-elimination of the
83 polar head, together with an allylic C-5 hydrogen atom (Lee *et al.*, 2010).

84 The use of transgenic plants, especially, represents a novel solution to the
85 challenges of studying the biochemical and ecological relevance of terpenes (Aharoni *et al.*
86 *et al.*, 2005). For instance, targeting FaNES1, a strawberry linalool/(*E*)-nerolidol synthase,
87 to the mitochondria resulted in the production of (*E*)-nerolidol and DMNT in transgenic
88 *Arabidopsis* plants (Kappers *et al.*, 2005). Based on the presence of
89 mitochondria-targeted farnesyl diphosphate (FDP) synthase and TPS (FaNES2, a
90 homologue of FaNES1) (Aharoni *et al.*, 2004), it was suggested that this cell
91 compartment might also contain a potential pool for sesquiterpene biosynthesis.
92 Transgenic plant approaches using TPSs are therefore useful to reveal novel
93 biosynthetic pathways of terpenes and deepen our understanding of their mechanisms.
94 Moreover, such manipulations of volatile blends may also be applicable in integrated
95 pest management strategies that employ volatiles attracting herbivore enemies in

96 so-called push–pull systems (Khan *et al.*, 2008). All these considerations taken together
97 indicate that genetic “gain” of particular terpenes is essential to fully exert the
98 ecological and agricultural functions of individual terpenes.

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

118 critical role of PITPS2 in the regulation of herbivore-induced formation of GL and TMTT.
119 This paper also addresses the issue of the complicated nature of indirect plant defenses
120 when transgenic plants are used in indirect pest control.

121

122 **Materials and Methods**

123 Plants and arthropods

124 Lima bean (*Phaseolus lunatus*) plants were grown in a greenhouse. Each individual
125 plant was grown in a plastic pot in a growth chamber at 25°C with a photoperiod of 16 h
126 (natural+supplemental light) for 2 weeks. Tobacco (*Nicotiana tabacum* cv. SR1) and
127 *Lotus japonicus* (ecotype Miyakojima MG-20) plants were grown in plastic pots in a
128 growth chamber at 25°C (16 h photoperiod at a light intensity of 80 $\mu\text{E m}^{-2} \text{s}^{-1}$) for 4-6
129 weeks. *T. urticae* was reared on kidney bean plants (*Phaseolus vulgaris*) in another
130 greenhouse under the same conditions. *P. persimilis* was obtained from a commercial
131 source (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands). *N.*
132 *californicus* was collected from *Pueraria lobata* plants infested with *Tetranychus*
133 *puerariicola* in a field at the National Agricultural Research Center in Ibaraki Prefecture,
134 Japan. These predators were reared on *T. urticae*-infested bean plants in a
135 climate-controlled room (25°C, 16 h photoperiod). Fertilized adult females 3–5 d after
136 the final molting were used for the bioassays. To prepare starved predators, the
137 predators were individually placed in sealed plastic tubes (1.5 ml), each containing a
138 drop of water (3 μl), in the laboratory for 24 h.

139

140 Chemical and herbivore treatment

141 For chemical treatment, JA (0.5 mM, pH 5.8–6.0, Wako Pure Chemical Industrials, Ltd.,
142 Osaka, Japan) in 2 mL of water was sprayed onto intact plants in plastic pots.
143 Alamehycin (0.1 μ M, Sigma-Aldrich, St. Louis, MO, USA) was applied to the petioles
144 of detached lima bean plantlets in aqueous solution. For herbivore treatment, a lima
145 bean plant and an *L. japonicus* plant were treated with 40 or 50 *T. urticae* adult females,
146 respectively. All treatments were carried out in a climate-controlled chamber at 25°C
147 (16 h photoperiod).

148

149 cDNA cloning

150 Total RNA was isolated and purified from leaf tissues using a Qiagen RNeasy Plant
151 Mini Kit and an RNase-Free DNase Set (Qiagen, Hilden, Germany). First-strand
152 cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA, USA),
153 oligo(dT)₁₂₋₁₈ primer, and 1 μ g of total RNA at 50°C for 50 min. For polymerase chain
154 reaction (PCR), primers for the *PITPS2* cDNA fragment were designed using partial
155 DNA sequences of an expressed sequence tag (EST) clone (annotation number:
156 CV540470) obtained from the TIGR *P. vulgaris* EST database:
157 http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=p_vulgaris. PCR was
158 performed with 2 min at 95°C; 35 cycles of 15 sec at 94°C, 30 sec at 55°C, and 60 sec
159 at 72°C. Further cloning of 5'- and 3'-ends was accomplished by rapid amplification of
160 cDNA ends (RACE) PCR using a First Choice RLM-RACE Kit (Ambion, Austin, TX,
161 USA) following the manufacturer's protocol.

162

163 Recombinant PITPS2 enzyme preparation and assay

164 For functional identification, cDNAs were amplified by PCR using *Pfu* DNA

165 Polymerase (Promega, Madison, WI, USA) with a set of primers for an open reading

166 frame (ORF) of *PITPS2*. The cDNA was subcloned into the pHis8-3 expression vector167 (Jez *et al.*, 2000). The recombinant vectors (pHis8.3-*PITPS2*) were transformed into168 *Escherichia coli* BL21-CodonPlus(DE3). The resultant bacterial strain was grown to169 $A_{600} = 0.5$ at 37°C in 5 ml of LB medium with kanamycin at 50 $\mu\text{g ml}^{-1}$. Cultures were170 induced with 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) and kept overnight

171 at 16 °C while being shaken at 200 rpm. Cells were pelleted by centrifugation and

172 resuspended in 250 μl of assay buffer (25 mM HEPES, pH 7.3, 12.5 mM MgCl_2 , 0.25173 mM MnCl_2 , 0.25 mM NaWO_4 , 0.125 mM NaF, 10 mM DTT, 10% glycerol).

174 Resuspended cells were broken by sonication. Cell extracts were clarified by

175 centrifugation and assayed for TPS activity with 50 μM geranyl diphosphate (GDP,

176 Echelon Biosciences Incorporated, Salt Lake City, UT, USA), FDP (Echelon

177 Biosciences Incorporated) or GGDP (Sigma-Aldrich). The assay mixture was covered

178 with pentane containing *n*-bromodecane (100 $\text{ng } \mu\text{l}^{-1}$), as an internal standard, to trap

179 volatile products. After incubation at 30°C for 1 h, the pentane layer was transferred to a

180 glass vial and analyzed. Extracts of *E. coli* transformed with expression vectors without

181 the TPS gene were used as controls following the above procedure. The enzymatic

182 reaction products were analyzed on a ThermoQuest/Finnigan TRACE GC 2000 with a

183 TRACE MS (Manchester, UK) equipped with an ECTM-5 capillary column (0.25 mm i.d.

184 x 15 m with 0.25-mm film, Alltech, Deerfield, IL, USA). Injection volume: 1 μ l; 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 *PITPS2* (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-*PITPS2* 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 T₁ 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. T₂ seeds harvested from each individual
205 T₁ plant that showed ca. 3:1 segregation ratio were tested for hygromycin-resistance

206 again. Both T₁ and T₂ plant lines were used for further chemical and gene expression
207 analyses, and homozygous T₃ 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 μ g 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 μ l from 10 μ l 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 *PIACT1* mRNA (GenBank
225 accession no. DQ159907), *LjTUB* (AB510590) or *NtACT* (GQ281246). Primers used
226 for this study are shown in Table S1.

227

228 Transient expression of green fluorescent protein (GFP) fusion proteins
229 Gateway Technology (Invitrogen) was used for the generation of pGWB451-*PITPS2*
230 transformation constructs, which consisted of *PITPS2* ORF cDNA bearing an N-terminal
231 fusion to *G3GFP* under the control of the cauliflower mosaic virus (CaMV) 35S
232 promoter (Nakagawa *et al.*, 2007). The resulting plasmid, pGWB451-*PITPS2*, was
233 transformed into *Agrobacterium tumefaciens* strain EHA101 by electroporation.
234 pGWB452, which expresses *G3GFP* under the control of the CaMV 35S promoter
235 (Nakagawa *et al.*, 2007), served as control. The bacteria were cultured in 50 ml of
236 Luria-Bertani (LB) medium/rifampicin/kanamycin/spectinomycin at 28°C for 36 h, and
237 1 ml of the cell culture was inoculated in 50 ml of LB/kanamycin/spectinomycin. Cells
238 were harvested by centrifugation and resuspended in 10 mM MES-NaOH, pH 5.6, 10
239 mM MgCl₂ and 150 mM acetosyringone (Sigma-Aldrich). The bacterial suspensions
240 were adjusted to OD₆₀₀ 1.0, incubated for 4 h at 28°C, and then infiltrated into leaves of
241 2-3-week-old lima bean plants using a needleless syringe. After 36-40 h, GFP
242 fluorescence was observed in lima bean leaves under a Nikon Eclipse C1 spectral
243 confocal laser scanner microscope (CLSM) with a 60x Plan Apo 1.40/oil objective
244 (Nikon Instruments, Tokyo, Japan). The microscope operates with two lasers: GFP was
245 excited at 488 with a krypton/argon laser, and chlorophyll autofluorescence was excited
246 at 647 nm with a HeNe-Laser. Emissions wave was collected through a 506 to 530 nm
247 band-pass filter (for GFP) and a 650 nm low-pass filter (for chlorophyll).
248
249 Volatile analysis

250 Headspace volatiles from potted plants were collected in a glass container (2 l) using
251 100 mg of Tenax-TA resin (20/35 mesh; GL Science, Tokyo, Japan) packed in a glass
252 tube (3.0 mm i.d., 160 mm length) in a laboratory room (25°C, light intensity of 80 μE
253 $\text{m}^{-2} \text{s}^{-1}$). Pure air gas ($\text{CO} < 1 \text{ ppm}$, $\text{CO}_2 < 1 \text{ ppm}$, $\text{THC} < 1 \text{ ppm}$) was drawn into the
254 glass bottle, and volatile compounds from the headspace of the bottle were collected
255 with Tenax-TA for 2 h at a flow rate of 100 ml min^{-1} . *n*-Tridecane (0.1 μg) as internal
256 standard was also added to the glass container. The volatile compounds collected were
257 analyzed by gas chromatography-mass spectrometry (GC-MS) according to the method
258 described by (Shimoda *et al.*, 2012). The headspace volatiles were identified and
259 quantified by comparing their mass spectra and retention times with those of authentic
260 compounds. All experiments were repeated four to seven times. DMNT and TMTT
261 were synthesized in the laboratory.

262

263 Non-volatile analysis

264 Leaves of 4-6-week-old *N. tabacum* (about 3 g each) or *L. japonicus* (about 0.5-1 g each)
265 plants were harvested and ground to a fine powder with a mortar and pestle under liquid
266 nitrogen. Ethyl acetate (1:5 w/v) spiked with 25 μg of internal standard (borneol,
267 Sigma-Aldrich) was added to each sample, and then the mixture was homogenized.
268 Extracts were transferred into a glass tube and centrifuged at 5000 *g* for 5 min at 4°C. The
269 resultant pellets were rinsed with the same volume of ethyl acetate and centrifuged. The
270 combined organic layers were subsequently adjusted to 500 μl under a nitrogen flow.

271 The extracts from *N. tabacum* and *L. japonicus* (1 μl and 5 μl , respectively) were
272 injected into a gas chromatograph (6890N, Agilent Technologies, Santa Clara, CA, USA)
273 coupled with a mass spectrometer (5973A, Agilent Technologies). Compounds were
274 separated on a Zebron ZB-5MS capillary column (7HG-G010-11, Phenomenex, Torrance,
275 CA, USA; stationary phase: 95% polydi-methyl siloxane - 5% diphenyl, length: 30 m,
276 inner diameter: 0.25 mm, film thickness: 0.25 μm) with the following temperature
277 program: 60°C for 5 min followed by a temperature rise at a 4°C min^{-1} rate up to 270°C
278 and 7°C min^{-1} rate to 290°C (held for 1 min). The carrier gas was He with a constant flow
279 of 1 ml min^{-1} , transfer line temperature to MSD was 280°C, ionization energy (EI) 70 eV,
280 and full scan range 50–320 m/z . GL was identified by comparison with an authentic
281 standard (Sigma-Aldrich) using the NIST mass spectral search software v2.0 with the
282 NIST 98 library. GL quantitation was assessed by GC-FID (6890N, Agilent
283 Technologies) with the same experimental procedures as described above.

284 In order to analyze terpenoid-sugar conjugates, the pellets obtained above were
285 extracted with 4 ml of citrate buffer (pH 5.2), transferred to a glass tube and centrifuged at
286 5000 g for 5 min at 4°C. Supernatants (about 3 ml) were collected in fresh glass tubes
287 and hydrolyzed enzymatically by adding 10 mg (about 60 U) of β -glucosidase (from
288 almonds, Sigma-Aldrich). The mixture was covered with pentane (5 ml) containing
289 borneol (25 μg), as an internal standard, to trap volatile products. After incubation at
290 37°C for 24 h, the pentane layer was transferred to a glass vial, reduced to a final
291 volume of 500 μl and analyzed using GC-FID and GC-MS (described above).
292 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 $80 \mu\text{E m}^{-2} \text{s}^{-1}$), 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**

337 Functional characterization of PITPS2

338 In order to verify the functional involvement of *PITPS2* in volatile biosynthesis, we
339 determined a full-length cDNA sequence for the gene from lima bean (GenBank
340 accession no. KC012520). The deduced nucleotide sequence of *PITPS2* encodes a
341 predicted protein of 569 amino acids that shares 78% identity and 83% similarity with a
342 *Glycine max* predicted (3S,6E)-nerolidol synthase (XP_003528418) and 66% identity
343 and 72% similarity with a *Medicago truncatula* linalool/(E)-nerolidol/GL synthase
344 (MtTPS3, AY766249) in the TPS-g group (Fig. S1). A functional assay of the
345 recombinant PITPS2 with prenyl diphosphatate (GDP, FDP or GGDP) as substrate
346 resulted in the production of the monoterpene linalool from GDP, the sesquiterpene
347 (E)-nerolidol from FDP and the diterpene GL from GGDP (Fig. 1). The recombinant
348 protein generated linalool as the predominant product, as well as (E)-nerolidol and GL
349 at approximately 82% and 16% of the rate of linalool. This ratio is different from the
350 composition of the product of the homologue MtTPS3 (linalool:(E)-nerolidol:GL =
351 5:100:65 (Arimura *et al.*, 2008). A control extract prepared from the
352 BL21-CodonPlus(DE3) strain transformed with a plasmid without the *PITPS2* cDNA
353 insert did not produce any terpene products (data not shown).

354

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

357 Using quantitative RT-PCR of RNA from lima bean leaves, we analyzed the
358 transcriptional levels of *PITPS2* 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 *PITPS2* transcripts at 2 h, and more dramatically at 6 and 24 h, after application.
362 Similarly, *PITPS2* 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 *PITPS2* activation on JA signaling (Fig. 2a).

366 Next, to test if the transcriptional profile of *PITPS2* 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 *PITPS2* 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 *PITPS2* 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 *PITPS2*

381 The subcellular localization of PITPS2-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 PITPS2 (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 *PITPS2* 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 *PITPS2*], NtMT [*N.*
393 *tabacum* expressing *MtTPS3*], LjPT [*L. japonicus* expressing *PITPS2*], and LjMT [*L.*
394 *japonicus* expressing *MtTPS3*]). Following selection for hygromycin resistance in the T₁
395 and T₂ plant lines, positive plants were grown and used for further experiments. All the
396 transgenic lines exhibited trans-gene (*PITPS2* 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: $\chi^2 =$
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: $\chi^2 = 3.127$,
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* ($G_p = 4.312$, $P < 0.05$, replicated
439 G-test, Fig. 6a) and TMTT from uninfested LjPT3 plants ($G_p = 7.312$, $P < 0.01$,
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) ($G_p = 0.450$, $P = 0.502$, 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 these 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 PITPS2

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 PITPS2 enables the conversion of three prenyl diphosphate substrates: GDP, FDP and
466 GGDP. PITPS2 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

469 characterized from rice, snapdragon, tomato, grape, and strawberry (reviewed in Tholl
470 *et al.* (2011)).

471 *In vitro* assays with the recombinant PITPS2 enzyme extracted from *E. coli*
472 showed that the monoterpene linalool is the predominant product, although the
473 sesquiterpene (*E*)-nerolidol and the diterpene GL are produced at 82% and 16% of the
474 levels of linalool, respectively (Fig. 1). Yet, transgenic *L. japonicus* plants expressing
475 *PITPS2* generated GL and its degradation product TMTT, but neither linalool nor
476 (*E*)-nerolidol. Therefore, it can be assumed that independent TPS provides linalool and
477 the precursor for DMNT formation in lima bean and *L. japonicas*. Moreover, differences
478 of the biochemical conditions between plant and microbial expression systems may in
479 many cases cause distinct product spectra, as shown in the catalysis of TPSs, where a
480 divalent metal ion such as Mg²⁺ or Mn²⁺ is required (Köllner *et al.*, 2004). The
481 heterologous expression of geraniol synthase (GES) from *Ocimum basilicum* in various
482 microbial (*Saccharomyces cerevisiae* and *E. coli*) and plant (*Vitis vinifera*, Arabidopsis,
483 and *Nicotiana benthamiana*) systems revealed that heterologous expression in various
484 plant and bacterial systems greatly influences the amount of the GES products in leaf
485 tissues or culture media, and the qualitative profile in the metabolically engineered *in*
486 *vivo* conditions (Fischer *et al.*, 2012). The functional properties of TPS are, therefore,
487 likely to depend not only on the enzyme's amino-acidic sequence but also on the
488 cellular compartment, especially when comparing between plant and bacterial systems.

489 More notably, genetic engineering of TPSs in plants does not always cause
490 production of substantial amounts of terpenes in transgenic plants, most probably

491 because of a lack of sufficient precursors in the protein-targeted cellular components
492 (Hohn & Ohlrogge, 1991; Wallaart *et al.*, 2001). This would hold true for both
493 transgenic *L. japonicus* and *N. tabacum* plants expressing MtTPS3 in the current study.
494 Similarly to PITPS2, MtTPS3 appears to be targeted to the plastids (Gomez *et al.*, 2005),
495 and *in vitro* assays have shown that MtTPS3 predominantly converts FDP to
496 (*E*)-nerolidol (Arimura *et al.*, 2008), whereas FDP, the precursor for sesquiterpenes, is
497 not sufficiently available in the plastid (Wu *et al.*, 2006). Alternatively, the failure of *de*
498 *novo* synthesis of MtTPS3 proteins and/or their folding is also a possible cause.

499

500 Degradation of GL leads to TMTT formation

501 GL, the PITPS2 product, appears to be converted to TMTT via an oxidative C-C bond
502 cleavage reaction in plants (Donath & Boland, 1994; Donath & Boland, 1995; Piel *et al.*,
503 1998), as proven using transgenic *L. japonicus* plants in the current study and transgenic
504 Arabidopsis plants in a previous study (Herde *et al.*, 2008). In Arabidopsis, the
505 herbivore-induced biosynthesis of TMTT appears to be catalyzed by CYP82G1, a P450
506 of the so-far uncharacterized plant CYP82 family (Lee *et al.*, 2010). Recombinant
507 CYP82G1 has shown narrow substrate specificity for GL and its C(15)-analog
508 (*E*)-nerolidol, which is converted to the respective DMNT (Lee *et al.*, 2010).
509 Notwithstanding, GL was not successfully converted to TMTT in any of the WT or
510 transgenic *N. tabacum* plants, implying a lack of potential conversion via a CYP82G1
511 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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603

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740

741 **Supporting Information**

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

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

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

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

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

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

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

752 **Table S1.** Primers used for this study.

753

754 **Figure legends**

755 **Fig. 1** Terpenes formed by the extracts containing the recombinant PITPS2 enzymes.

756 The PITPS2 products from assays of the extract prepared from the
757 BL21-CodonPlus(DE3) strain transformed with the recombinant vector
758 (pHis8.3-*PITPS2*), with GDP (a), FDP (b) or GGDP (c) as substrate, are illustrated. IS,
759 internal standard. (d) Absolute values for the PITPS2 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 *PITPS2* (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-*PITPS2* (35S

769 promoter-*PITPS2-G3GFP*) and pGWB451-*PITPS2*. Gateway, the Gateway cassette;

770 *P35S*, 35S promoter; *Tnos*, nopaline synthase terminator. (d) Subcellular localization of

771 PITPS2-GFP fusion protein in lima beans. Leaves were transformed with the control

772 plasmid pGWB452 or pGWB451-*PITPS2* 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.

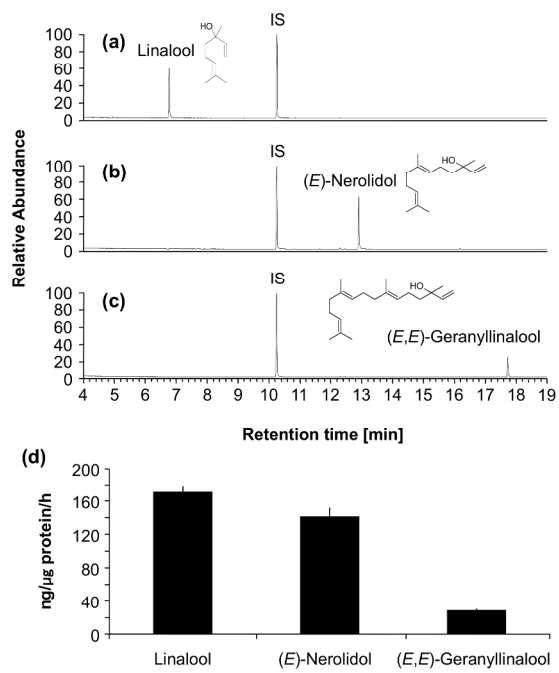


Fig. 1

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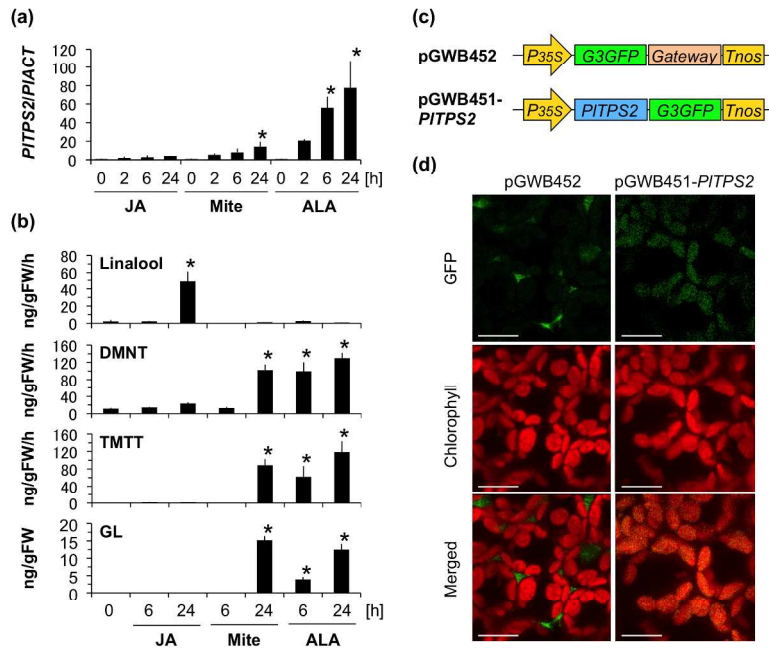


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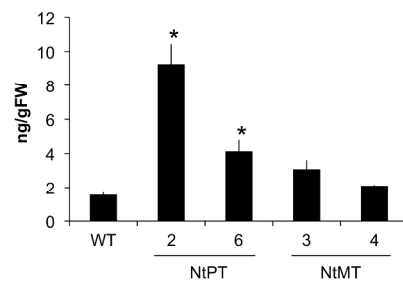


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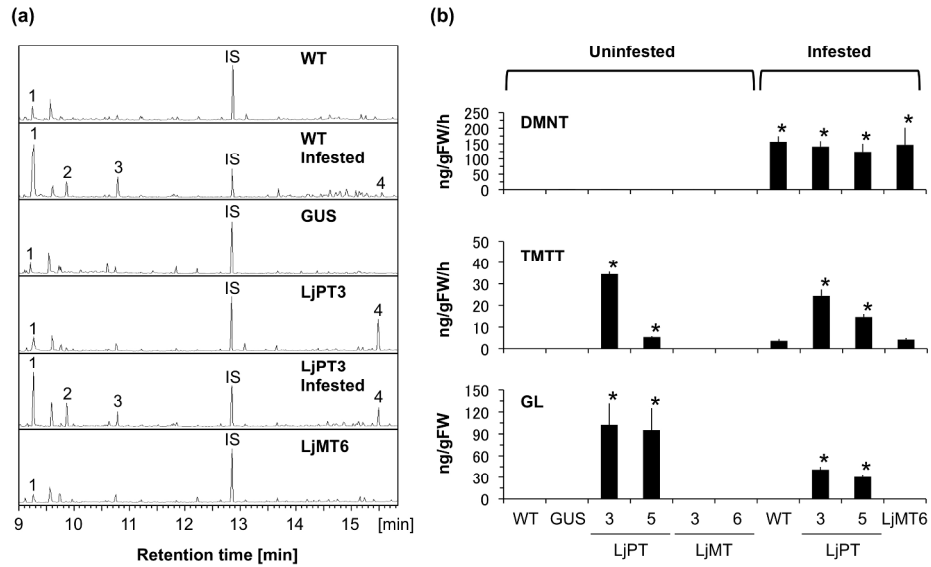


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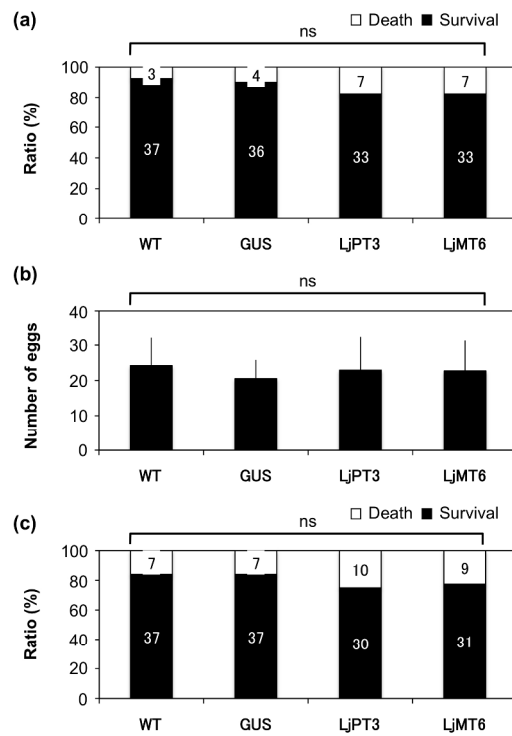


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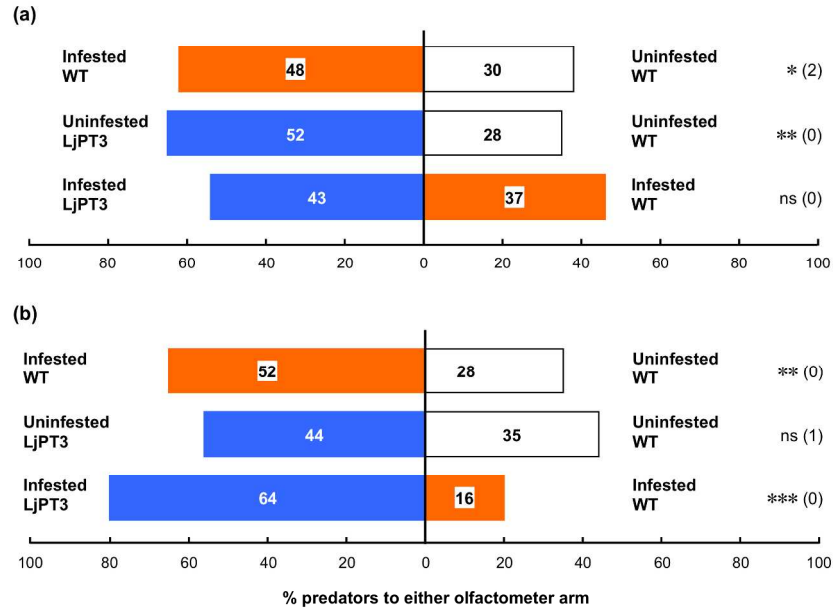


Fig. 6

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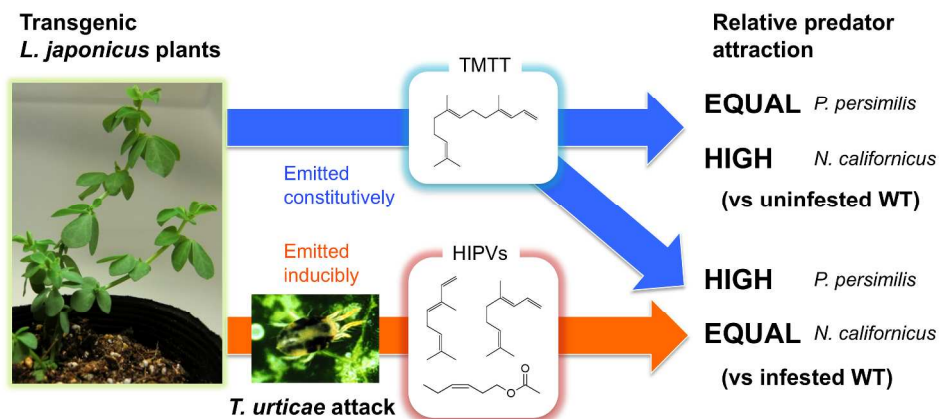


Fig. 7

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