



Three-step pathway engineering results in more incidence rate and higher emission of nerolidol and improved attraction of *Diadegma semiclausum*

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ABSTRACT

The concentration and ratio of terpenoids in the headspace volatile blend of plants have a fundamental role in the communication of plants and insects. The sesquiterpene (*E*)-nerolidol is one of the important volatiles with effect on beneficial carnivores for biologic pest management in the field. To optimize *de novo* biosynthesis and reliable and uniform emission of (*E*)-nerolidol, we engineered different steps of the (*E*)-nerolidol biosynthesis pathway in *Arabidopsis thaliana*.

Introduction of a mitochondrial nerolidol synthase gene mediates *de novo* emission of (*E*)-nerolidol and linalool. Co-expression of the mitochondrial *FPS1* and cytosolic *HMGR1* increased the number of emitting transgenic plants (incidence rate) and the emission rate of both volatiles. No association between the emission rate of transgenic volatiles and their growth inhibitory effect could be established. (*E*)-Nerolidol was to a large extent metabolized to non-volatile conjugates.

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1. Introduction

An important issue in ecology is to understand how interactions among individual organisms influence food webs and community dynamics. Plants are challenged by a wide range of herbivorous insects and have developed a multitude of defence strategies, including pre-existing physical and chemical barriers, tolerance mechanisms and induced defences that are activated upon attack, such as the biosynthesis of secondary metabolites. In induced direct defence, plant-herbivore interaction is affected in various ways such as through the production of repellents, toxins and by hindering digestion (Vasconcelos and Oliveira, 2004; Wittstock and Gershenzon, 2002). Plants can also promote the efficiency of the second trophic level including predators and parasitoids of their enemies by using the so called induced indirect defence (Gatehouse, 2002).

This indirect induced defence involves biosynthesis of a blend of plant volatiles that attract antagonists of plant enemies upon herbivory; a phenomenon called “cry for help” (Dicke and

Baldwin, 2010). This blend of herbivore induced plant volatiles (HIPV) can be complex and herbivore-specific, sometimes consisting of hundreds of compounds (Mumm and Dicke, 2010). The majority of compounds within the HIPV of many plant species belong to the terpenoids such as (3*S*)-(*E*)-nerolidol, (*E*)-4,8-dimethyl-1,3,7-nonatriene ((*E*)-DMNT), (*E*)-β-ocimene and (*E*)-α-farnesene. Other biochemical groups present in HIPV are green leaf volatiles, benzenoids and sulphur- or nitrogen-containing compounds. In the past decade, molecular genetic tools have been used to investigate the signalling pathways leading to HIPV emission and their effect on the attraction of carnivorous arthropods (Halitschke and Baldwin, 2003; Kessler and Baldwin, 2004; Snoeren et al., 2009; Van Poecke and Dicke, 2004). In addition, molecular insight into the biosynthesis of HIPVs has allowed the genetic modification of the production of these volatile compounds. These modified plants were successfully used to study the effect of these volatiles on the behaviour of carnivorous arthropods (Aharoni et al., 2005; Bouwmeester et al., 2003; Degenhardt et al., 2003; Kappers et al., 2005; Loivamaki et al., 2008; Schnee et al., 2006; Tholl et al., 2005). These studies demonstrate the effectiveness of HIPV pathway engineering to improve biological control of above ground as well as below ground pests (Degenhardt et al., 2009).

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The C15-sesquiterpene (*E*)-nerolidol and the C11-homoterpene (*E*)-DMNT are examples of HIPVs detected in the odour blend of many plant species after herbivory including cucumber, lima bean, maize, tomato and some *Arabidopsis thaliana* accessions (Ament et al., 2004; Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000; Snoeren et al., 2010). *De-novo* emission of these volatiles in the headspace of transgenic *A. thaliana* improves the attraction of predatory mites to *A. thaliana* (Kappers et al., 2005).

FaNES1 (*Fragaria X ananassa* nerolidol synthase1) is a sesquiterpene synthase that mediates *in vitro* biosynthesis of (*E*)-nerolidol from FPP (farnesyl diphosphate) and the monoterpene linalool from GPP (geranyl diphosphate) (Aharoni et al., 2003). The failure to produce appreciable amount of (*E*)-nerolidol with cytosolic FaNES1 and plastidic was speculated to be due to the lack of available FPP (the precursor of sesquiterpenoids) in the cytosol or plastids (Aharoni et al., 2003). Therefore, Kappers et al. (2005) targeted FaNES1 to the mitochondria as *A. thaliana* possesses a long isoform of FPP synthase with a mitochondrial targeting signal (FPS1L) (Cunillera et al., 1997). Hereto, the CoxIV signal peptide from yeast (Kohler et al., 1997) was used. Transgenic *A. thaliana* plants harbouring the CoxIV-FaNES1 construct produced clearly detectable amounts of (*E*)-nerolidol and (*E*)-DMNT in their odour blend (Kappers et al., 2005). However, transgenic plants displayed some growth retardation of the rosette (Aharoni et al., 2003).

Considering the potential of metabolic engineering to enhance a plant's indirect defence, a consistent emission of engineered HIPVs and uniform growth of plants are important traits and growth retardation is hence an unwanted side-effect. One could speculate that the introduction of CoxIV-FaNES1 diverts the mitochondrial FPP that is also required for ubiquinone and heme A biosynthesis and consequently causes growth inhibition. In order to reduce the harmful effects on plant growth and development, the levels of specific intermediates of the pathway, particularly those at branch-point positions should be controlled (Manzano et al., 2004). Knowing that an increase in precursor supply can elevate the level of terpenoids in plants in the presence of the corresponding terpene synthase (Aharoni et al., 2005; Chen et al., 2000; van Herpen et al., 2010), the undesirable phenotypes can possibly be diminished by over-expressing FPS1L to provide a larger pool of FPP in the mitochondria. As isopentenyl diphosphate (IPP, the substrate for FPS1L) might be also a rate limiting factor, over-expression of *A. thaliana* HMGR1S (3-hydroxy-3-methylglutaryl coenzyme A reductase 1 short isoform) that encodes an earlier step in the mevalonate pathway and has been suggested to be rate limiting (Chappell et al., 1991) could further reduce the growth retardation. Over-expression of HMGR1S has already been shown to prevent necrosis in leaves of FPS1S (short isoform of FPS which is cytosolic) overexpression lines (Manzano et al., 2004) and premature senescence in detached leaves of FPS1L (long isoform of FPS, which is mitochondrial) overexpression lines under continuous illumination (Manzano et al., 2006). Furthermore, transient co-expression of tHMGR (truncated form of HMGR) and FPS with amorphaadiene synthase has been shown to improve amorphaadiene biosynthesis in *Nicotiana benthamiana* (van Herpen et al., 2010).

In this study, two rate-limiting genes of the mevalonate pathway, HMGR1S and mitochondrial FPS1L were co-expressed with mitochondrial nerolidol synthase. The volatile compounds of the headspace and non-volatile compounds in the semi-polar extracts of wild type and transgenic lines were analysed. The effect of the volatile blends from intact and herbivore-induced transgenic plants on *Diadegma semiclausum*, a beneficial parasitoid wasp against the Brassicaceae specialist caterpillar *Plutella xylostella*, is investigated. The results of this research are used to select the most efficient transformation strategy to produce a

reliable and efficient amount of transgenic volatiles for optimal indirect insect defence.

2. Materials and methods

2.1. Generation of transgenic plants

The CoxIV-FaNES1 construct originally used for transformation in Kappers et al. (2005) was sub-cloned into pCambia vector and used to produce *A. thaliana* Col-3 transgenic plants. Subsequently, produced single CoxIV-FaNES1 insertion plants and homozygous transgenic plants from independent transformation events harbouring single T-DNA insertions of either HMGR1S (hydroxymethylglutaryl CoA reductase1 isoform) (Manzano et al., 2004) and FPS1L (farnesyl diphosphate synthase1 long isoform) (Manzano et al., 2006) were crossed and selfed to produce independent transgenic lines of *A. thaliana* Col-3 with single, double or triple transgenes (Table 1). F3-progenies of crossed transgenic lines were sown on selective plates. The transgenes were all under the control of the constitutive CaMV 35S promoter.

Seeds of wild type and transgenic lines were surface-sterilized, germinated on autoclaved 0.9% Daishin agar (Duchefa, The Netherlands) with 0.5 MS+vitamins (pH > 6) (Duchefa, The Netherlands) and 10 µg ml⁻¹ BASTA (only transgenics) in 14 cm Petri dishes and incubated at 21 ± 2 °C for ca. 10 days under a L8:D16 photoperiod with 80–110 µmol m⁻² s⁻¹ PPF. Four-leaf seedlings were transplanted into potting soil (Lentse potgrond BV, Lent, Netherlands, heat-sterilized in 80 °C overnight prior to use) and grown under the same conditions as above. Plants were watered twice a week. To prevent infestation by sciarid larvae, the soil was treated weekly with *Steinernema feltiae* entomopathogenic nematodes (Koppert B.V. Berkel en Rodenrijs, the Netherlands). Six to eight weeks after sowing, fully grown vegetative plants were used for experiments.

2.2. Herbivores and parasitoids

The herbivore *Pieris rapae*, the small cabbage white, was reared on Brussels sprouts (*Brassica oleracea* var. *gemmifera*, cv Cyrus) in a growth chamber (L16:D8; 20 ± 2 °C and 70% RH). For herbivory induction, *Arabidopsis* plants were infested by equally distributing 20 s-instar *P. rapae* larvae per plant over the fully expanded leaves 24 h prior to the experiment. Herbivorous larvae were removed from plants before behavioural assays.

Table 1

Wild type (WT) and transgenic *Arabidopsis thaliana* lines in this study. All transgenes were under the control of the constitutive 35 s Cauliflower Mosaic Virus promoter.

Line	Group	Harbored transgenes
WT	Col-3 (WT)	–
A	FPS1L	FPS1L
B	HMGR1S::FPS1L	HMGR1S + FPS1L
C	HMGR1S::FPS1L	HMGR1S + FPS1L
D	COX	CoxIV ^a -FaNES1
E	COX	CoxIV ^a -FaNES1
F	COX	CoxIV ^a -FaNES1
G	COX	CoxIV ^a -FaNES1
H	COX+	FPS1L + CoxIV ^a -FaNES1
I	COX+	FPS1L + CoxIV ^a -FaNES1
J	COX+	FPS1L + CoxIV ^a -FaNES1
K	COX++	HMGR1S + FPS1L + CoxIV ^a -FaNES1
L	COX++	HMGR1S + FPS1L + CoxIV ^a -FaNES1
M	COX++	HMGR1S + FPS1L + CoxIV ^a -FaNES1

^a CoxIV: mitochondrial signaling peptide from yeast.

Parasitoid wasps, *D. semiclausum*, were reared as described (Bukovinszky et al., 2005). Emerging wasps were provided ad libitum with water and honey and are referred to as 'naïve' wasps as they had neither been exposed to plant material, nor obtained oviposition experience. The parasitoid is known to be attracted to the volatiles emitted by *P. rapae*-infested *A. thaliana* Col-0 (Loivamaki et al., 2008; Snoeren et al., 2010).

2.3. Headspace trapping and analysis of volatiles by GC–MS

In the first headspace experiment, 12 plants of each of the 14 genotypes (wild type and 13 lines) (Table 1) in vegetative stage (6 weeks) were sampled in groups (replicates) of 4 plants with similar rosette sizes, over a time period of four days. The headspace of each group was sampled for 4 h between 11:00 am and 15:00 pm. In a second headspace experiment, single plants of each of the 14 genotypes, in vegetative stage with equal rosette sizes were sampled over a time period of three weeks. In this experiment, headspace was sampled for 1.5 h between 11:00 am and 13:00 pm. For headspace trapping, transgenic plants were carefully placed in a 2.5 l glass jar and the jar was closed with a Viton-lined glass lid having an inlet and outlet. Inlet air was filtered by passing through stainless steel cartridges (Markes, Llantrisant, UK) containing 200 mg Tenax TA (20/35 mesh, Grace-Alltech, Deerfield, USA).

Headspace trapping was performed under the same conditions as the growing conditions. Collection of volatiles started 15 min after placing the plant(s) in the jar by sucking air out of the jar at a rate of 80 ml min⁻¹ through the cartridge. After the collection period, Tenax cartridges were dry-purged with nitrogen at 30 ml min⁻¹ for 20 min at ambient temperature to remove water before thermodesorption of the trapped volatiles.

Headspace samples were analysed with a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, USA) connected to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, USA) quadrupole mass spectrometer. Volatiles were desorbed from the cartridges using thermal desorption at 250 °C for 3 min (Model Ultra Markes Llantrisant, UK) with a Helium flow of 30 ml min⁻¹, focused (0 °C) on electronically-cooled sorbent trap (Unity, Markes, Llantrisant, UK). Volatiles were transferred splitless to the analytical column (Rtx-5 ms, 30 m, 0.25 mm i.d., 1.0 µm film thickness, Restek, Bellefonte, USA) by rapid heating of the cold trap to 250 °C. The GC was held at an initial temperature of 40 °C for 3.5 min followed by a linear thermal gradient of 10 °C min⁻¹ to 280 °C, which was held for 2.5 min with a column flow of 1 ml min⁻¹. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 45 to 400 *m/z* with a scan rate of 3 scans s⁻¹.

Volatile compounds were identified using the deconvolution software AMDIS (NIST, USA) and using their retention index and mass spectrum. The retention indices and spectra were compared with those of authentic standards (Sigma Aldrich Chemie, Germany, for (*E*)-nerolidol and linalool), data in the literature (Adams, 1995; Rohloff and Bones, 2005; Snoeren et al., 2010), NIST 2005 and an in-house mass spectral library and The Pherobase (<http://www.pherobase.com/database/kovats/kovats-index.php>) on a column with (5%-Phenyl)-methylpolysiloxane stationary phase or equivalent. NIST MS Search v.2.0 was used for *in-silico* mass spectrum comparisons.

The amount of volatiles in the first headspace experiment was estimated semi-quantitatively by calculating the peak area of the characteristic *m/z* (Supplementary Table 1) using the Quan Browse application of XCalibur and normalized by the fresh weight of the rosettes. Emitted amounts of (*E*)-nerolidol and linalool in the headspace of individual plants in the second experiment were quantified

using characteristic *m/z* for each compound (69 and 93 for (*E*)-nerolidol and linalool, respectively).

2.4. Metabolite analysis in leaf extracts using LC–MS

Hundred mg of flash frozen shoot material of each sample was powdered and mixed with 300 µl of ice-cold methanol acidified with 0.1% (v/v) formic acid. After vortexing for 5 s, sonication for 15 min and centrifugation (2500 rpm) for 10 min, the extracts were filtered through syringe filters (Minisart SRP 4, 0.45 µm, Sartorius Stedim Biotech) and collected in glass vials. Five µl of the filtered extract (150 µl) was injected and analysed by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC–QTOF–MS) operated in the negative ionization mode. This analytical system consisted of a Waters Alliance 2795 HPLC connected to a Waters 2996 PDA detector and subsequently to a QTOF Ultima V4.00.00 mass spectrometer (Waters, MS technologies, UK). A C18 pre-column (2.0 × 4 mm, Phenomenex, USA) was coupled to an analytical column (2.0 × 150 mm, 100 Å, particle size 3 µm, Phenomenex, USA) and degassed eluent A (1:1000 formic acid: ultrapure water) and eluent B (1:1000 formic acid: acetonitrile) were pumped at 0.19 ml min⁻¹ into the HPLC system. The gradient was increased linearly from 5% to 35% eluent B in 45 min. The column was washed and equilibrated for 15 min before the next injection. A collision energy of 10 eV was used for full-scan LC–MS in the range of *m/z* 100 to 1500. Leucine enkephalin ([M–H][–] = 554.2620) was used as a lock mass for online mass calibration.

2.5. Data analysis and in-silico identification of reconstructed metabolites

LC–MS acquired data were processed using MetAlign (Lommen, 2009) for noise elimination and alignment of the data points. LC–MS and GC–MS data were normalized with the fresh weight of the analysed samples and subsequently Log10 transformed and scaled symmetrically by the standard deviation using CANOCO (Smilauer, 2003). Detrended correspondence analysis (DCA) was used to check the length of the gradient (*L*) and due to an *L* < 4, the linear ordination techniques, principal component analysis (PCA) and redundancy analysis (RDA) were selected to visualize variation across chromatograms and correlations between metabolites using CANOCO (Smilauer, 2003). The procedure of Houshyani et al. (2011) was followed for putative identification of selected LC–MS metabolites. Assignment and screening of molecular formulas were done by using the Seven Golden Rules (Kind and Fiehn, 2007). PASW statistics 17 from SPSS was used for ANOVA and other mean comparisons as well as correlation analysis.

2.6. Behavioural assays using parasitoid wasps

The effect of the volatile blend of transgenic lines on parasitoid behaviour was compared with that of wild type plants using a closed-system Y-tube olfactometer as described before (Bukovinszky et al., 2005). In short, filtered air was led through activated charcoal and split into two air streams (4 l min⁻¹) that were led through five-litre glass vessels containing the odour sources (4 plants with similar rosette size in each vessel). The olfactometer was illuminated with artificial light from above at an intensity of 60 ± 5 µmol m⁻² s⁻¹ PPFD on the surface of the desk. All experiments were conducted in a ventilated room (20 ± 2 °C). To assess the attractiveness of (*E*)-nerolidol in parasitoid behaviour, 96% (*E*)-nerolidol (Sigma-Aldrich Chemie, Germany) released from a

glass capillary was compared with clean air in the same experimental setup.

Naive, 3–7 days-old female *D. semiclausum* were individually introduced into the Y-tube olfactometer using a glass tube. Upon release in the olfactometer, parasitoid behaviour and parasitoid choice for one of the two odour sources was assessed. Parasitoids that did not choose for any of the two arms of the olfactometer within five minutes after release, or chose an arm but did not make a final choice within ten minutes after release were considered as non-responding individuals, and were excluded from statistical analysis. Odour sources were interchanged after every five parasitoids to compensate for any unforeseen asymmetry in the set-up.

Parasitoid preference for odour sources was statistically analysed using a Chi-square test (null-hypothesis: parasitoids have the same preference for the two odour sources).

3. Results

3.1. Phenotypic changes in transgenic lines

Average plant weights showed a mixture of retarded, accelerated and normal growing habits for the transgenic lines under short day (L8:D16) growing conditions. Fresh weight of some of the transgenic lines differed significantly ($\alpha=0.05$) from that of the wild type Col-3 (Fig. 1, experiment 1). The observed differences were not associated with the introduced *FaNES1* gene product as the significant smallest lines were not among the highest (*E*)-nerolidol/linalool (derivatives) producers and vice versa (Fig. 1, experiment 1). Lines *E* (COX) and *J* (COX+) had on average a larger rosette compared to the wild type and line *C* (*HMGR1S::FPS1L*) and *D* (COX) that were not emitting (*E*)-nerolidol and line *I* (COX+) were significantly lighter than wild type plants; Lines *F*, *H*, *K*, *L* and *M* tend to be lighter on average

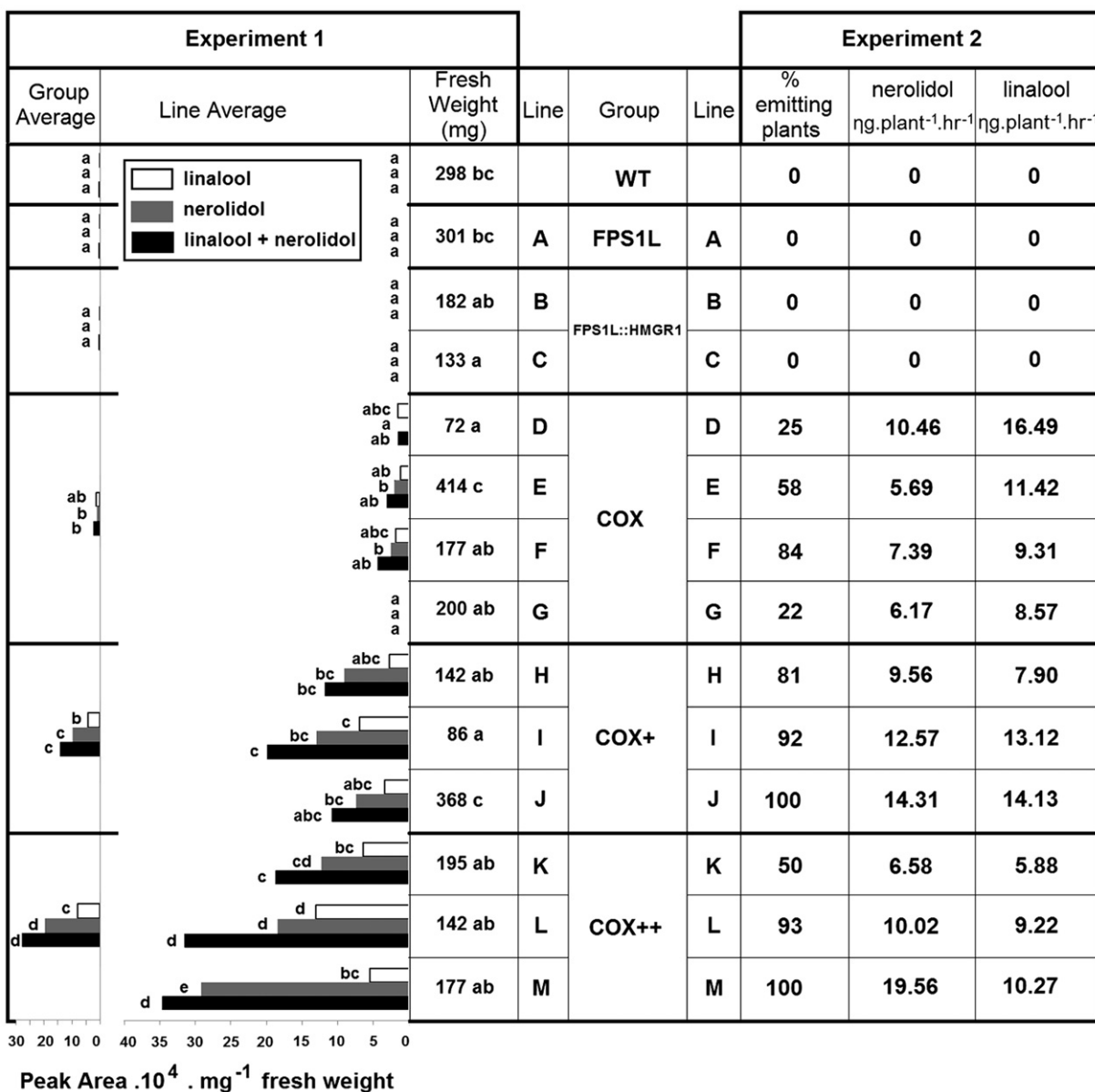


Fig. 1. Linalool and (*E*)-nerolidol production by wild type (WT) and transgenic lines in two experiments. Experiment 1 compares average fresh weight and linalool and (*E*)-nerolidol production of transgenic lines based on the peak area per mg fresh weight of the examined plants. Lower case letters indicate statistical comparison and grouping of the genotypes based on the corresponding parameter. Sharing the same letter indicates non-significant difference between the corresponding genotypes (ANOVA in conjunction with Tukey's test, $\alpha=0.05$). Experiment 2 compares transgenic lines based on the percentage of examined plants that emitted the transgenic volatile and the average quantity of linalool and (*E*)-nerolidol in emitting plants in ng per plant per hour. No significant difference was observed between transgenic volatile emitters in experiment 2. COX, CoxIV- *FaNES1* lines; COX+, FPS1L:: CoxIV-*FaNES1* lines and COX++, HMGR1S::FPS1L:: CoxIV-*FaNES1* lines (CoxIV, the mitochondrial signal).

(Fig. 1, experiment 1). These results were reproducible to some extent as in an independent experiment under the same growing conditions with 4 week old lines *I* (COX+, 170 mg) and *L* (COX++, 120 mg), they weighed significantly less than Col-3 (341 mg) (ANOVA, Tukey, $n=7$, p -value < 0.01 for both comparisons), while there was no significant difference between the two transgenic lines (p -value=0.137).

3.2. Effect of pathway engineering on the emission of linalool and (*E*)-nerolidol

In the first headspace experiment, 4 plants of each transgenic line were combined. The headspace of lines *A* (*FPS1L*), *B* and *C* (*HMGR1S::FPS1L*) was similar to the headspace of wild type plants as they produced neither linalool nor (*E*)-nerolidol (Fig. 1, experiment 1). Emission of (*E*)-nerolidol was detected in transgenic lines *E* and *F* (COX, *CoxIV-FaNES1* expressing lines). Linalool was present in 3 of the 4 analysed COX transgenic lines (*D*, *E* and *F*), while it was absent in the wild type (Fig. 1, experiment 1).

Addition of *FPS1L* to *FaNES1* lines (COX+) increased the emission of both linalool and (*E*)-nerolidol (Fig. 1, experiment 1), which was significantly higher in line *I* compared with COX lines *D*, *E*, *F* and *G* (Fig. 1, experiment 1). Lines *H* and *J* did not differ significantly from COX lines in emission. The triple construct COX++, overexpressing *HMGR1S* in combination with *FPS1L* and *CoxIV-FaNES1* also increased the average emission of both linalool and (*E*)-nerolidol (Fig. 1, experiment 1). Transgenic lines *L* and *M* showed significantly higher emission of linalool and (*E*)-nerolidol, respectively, compared with COX+ lines (Fig. 1, experiment 1).

In a second headspace experiment the headspace of individual plants from each transgenic line was analyzed. In two out of four analysed COX lines, less than half of the analysed plants emitted (*E*)-nerolidol and linalool (Fig. 1, experiment 2) at a level above the detection limit during the period of headspace trapping (1.5 h). Emitting plants within the COX group did not differ in quantities of (*E*)-nerolidol and linalool emitted by COX+ and COX++ plants (Fig. 1, experiment 2). The percentage of emitting plants in line *F* (COX) was comparable with the percentages of emitting plants in the COX+ and COX++ groups (> 80%) (Fig. 1, experiment 2). Line *F* was the highest emitter within the COX group just as in the first headspace experiment (Fig. 1, experiment 2).

More than 80% of the examined plants of both COX+ and COX++ lines emitted (*E*)-nerolidol and linalool, except for line *K*, where only 50% of the plants showed emission (Fig. 1, experiment 2). The average amounts of emitted (*E*)-nerolidol and linalool did not differ significantly between lines and varied across plants from 0.3 to 43 ng plant⁻¹ h⁻¹ for (*E*)-nerolidol and 0.3 to 40 ng plant⁻¹ h⁻¹ for linalool, respectively (Fig. 1, experiment 2).

The amount of (*E*)-nerolidol and linalool emitted by producing plants (Fig. 1, experiment 2) showed a significant correlation ($R=0.67$, p -value < 0.01). As stated above, the emission of (*E*)-nerolidol and linalool showed no significant differences between transgenic lines possibly due to the high variation. However, a chi-square test indicated that the assumption of transgenic volatile production by all COX plants was violated (p -value=0.002) while this assumption did hold for the COX+ and COX++ lines (p -value=0.92 and 0.28, respectively).

(*E*)-DMNT was clearly present in a number of COX, COX+ and COX++ plants (data not shown), but due to inconsistency in emission and high level of noise in the corresponding retention region we were unable to (semi) quantify the corresponding peak for further analysis.

3.3. Multivariate analysis of the headspace volatiles

To examine the overall effect of the introduced genes on the headspace profile, multivariate data analysis and ANOVA were

performed on headspace data of the first experiment. Due to a low signal/noise ratio in the analysis, we initially browsed profiles and searched for known compounds using previous studies on volatile compounds of *A. thaliana* (Rohloff and Bones, 2005; Snoeren et al., 2010). This resulted in a dataset of semi-quantitative data of 53 volatile compounds representing various biochemical groups of compounds (Supplementary Table 1). The retention time and characteristic mass of each compound was used for *in-silico* quantification of the corresponding peak area (semi-quantified data).

RDA was used to pinpoint those volatile compounds that could be important for differentiating the headspace volatile blend of transgenic lines from that of the wild type. Subsequently, any overlap with the list of significantly different volatile compounds (ANOVA, $\alpha=0.05$) was checked. In the RDA of semi-quantified data, wild type and transgenic lines (genotype) and biological replicates (biological variation) were set as explanatory variables for the observed variation in the data. Genotype and biological variation together explained 53.5% of the variation in the data. The RDA plot with the first (explaining 19.6% of the variation) and second (15.5%) ordinate showed a clear separation between lines based on the abundance of (*E*)-nerolidol and linalool (Fig. 2A). *FPS1L* and *HMGR1S::FPS1L* and two COX lines (*A*, *B*, *C*, *D* and *G*) without (*E*)-nerolidol in their headspaces were clustered with wild type samples on one side of the ordination diagram. Volatiles that fit more than 60% (arbitrary threshold) to the ordination model are shown in Fig. 2A with linalool and (*E*)-nerolidol having the largest contribution to the observed separation between lines, which is indicated by their direction.

ANOVA on the semi-quantified data showed that only six of the annotated volatiles differ significantly between the lines ($\alpha=0.05$): 4-methyl-2-pentanone, 3-methyl-3-butenenitrile, 3-butenyl isothiocyanate, (*E*)- β -ocimene, linalool and (*E*)-nerolidol. From this set of volatiles only linalool and (*E*)-nerolidol differed significantly between (*E*)-nerolidol producing and non-producing lines and appeared on the generated RDA plot (Fig. 2A). A similarity matrix with all significantly different volatiles showed no correlation between the abundance of linalool or (*E*)-nerolidol and other significantly different volatiles (data not shown). Excluding linalool and (*E*)-nerolidol from the dataset shortened the distance between (*E*)-nerolidol producing and non-producing lines as illustrated by the RDA diagram (Fig. 2B). Volatiles that fit more than 55% to the ordination model (arbitrary threshold, using a 60% threshold resulted in only one compound) and therefore important for the observed configuration on the ordination plot are pinpointed. These volatiles showed no significant difference across the lines (except for 4-methyl-2-pentanone). A PCA on the same dataset showed no clusters based on known structures (groups or lines) (Fig. 2C). All above observations suggest no major changes in the semi-quantified volatile profiles as a consequence of the introduction of the transgenes, except for the presence of (*E*)-nerolidol and higher quantities of linalool.

3.4. Analysis of conjugates of transgenic volatiles

To study metabolization of the transgenic volatiles *in planta*, methanol extracts of leaves of transgenic lines from the first experiment were analysed by LC-QTOF-MS. Biological replicates of extraction were the same as the replicates of the headspace trapping experiment. We followed a data integration and multivariate data analysis approach to search for any correlation between the analysed volatiles in the headspace and the analysed metabolites within the leaf. An RDA plot with headspace GC-MS data explaining the observed variation in leaf LC-MS data illustrated the relationship between volatile and non-volatile metabolites. For eight of the

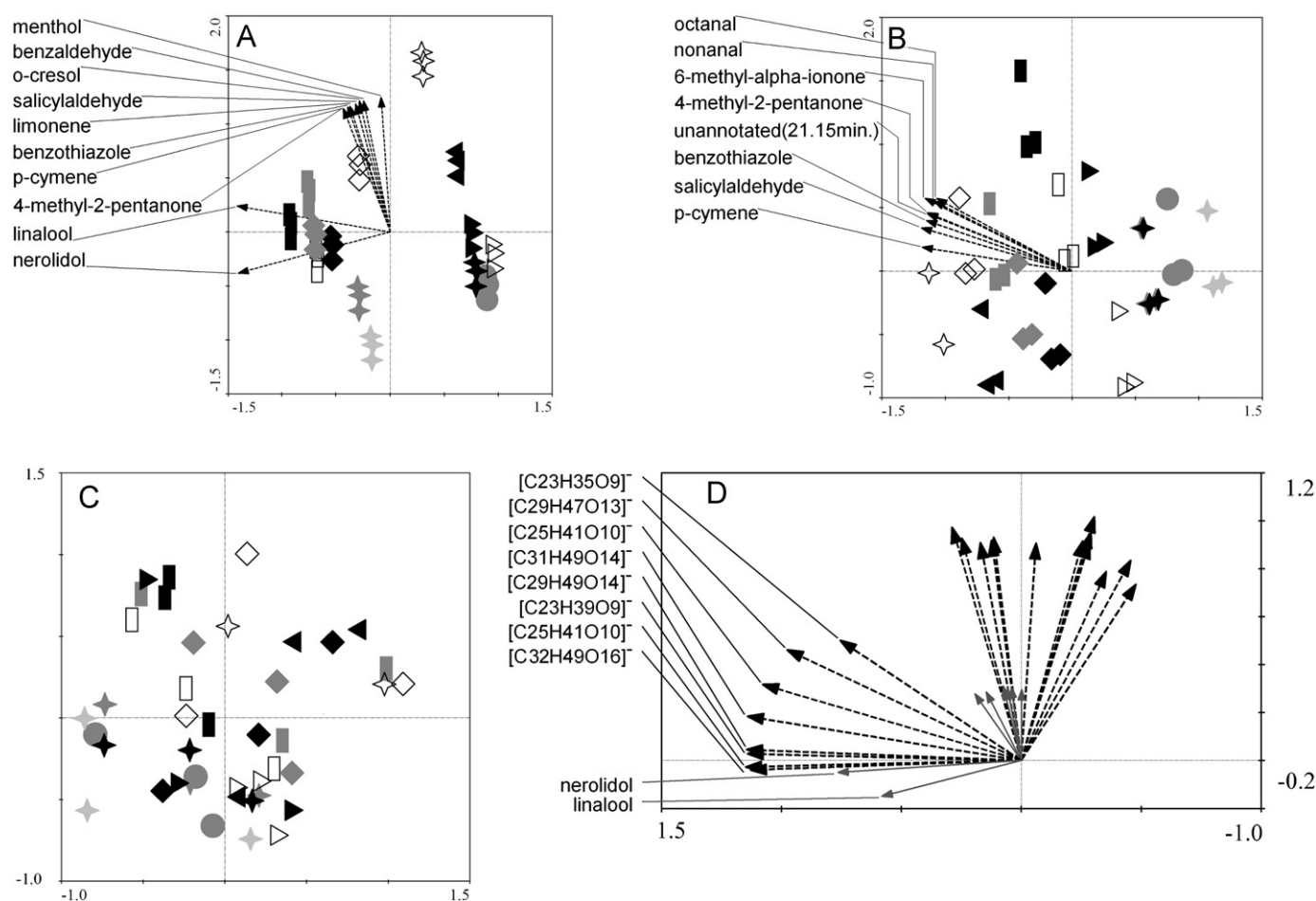


Fig. 2. Ordination plots by (1) redundancy analysis (RDA) using semi-quantified data including (A) and excluding (B) linalool and (E)-nerolidol. Transgenic lines (genotype) and biological replicates (biological variation) were set as explanatory variables for the observed variation in the peak areas. Marked metabolites are putatively identified volatiles fitting more than 60% (A) or 55% (B) to the ordination model and are hence important for the observed clustering of samples; (2) principal component analysis (PCA) using the semi-quantified data excluding linalool and nerolidol from the data set (C); (3) RDA showing the relationship between the non-volatile metabolites (dotted lines) that fitted more than 30% to the ordination diagram and head space volatiles (solid lines) that correlated between < -0.3 and > 0.3 with the ordination axes (D). Arrows pointing in the same direction indicate positive correlation and vice versa. Some of the proposed elemental compositions for non-volatile metabolites are indicated on the left. To focus on transgenic associated compounds only strongly correlated metabolites with linalool and (E)-nerolidol were annotated. ● WT (wild type), ▲ A (FPS1L), ▼ B (HMGR1S::FPS1L), ► C (HMGR1S::FPS1L), ☆ D (COX), ★ E (COX), ★ F (COX), ★ G (COX), ◇ H (COX+), ◆ I (COX+), ◆ J (COX+), □ K (COX++), ■ L (COX++), ■ M (COX++).

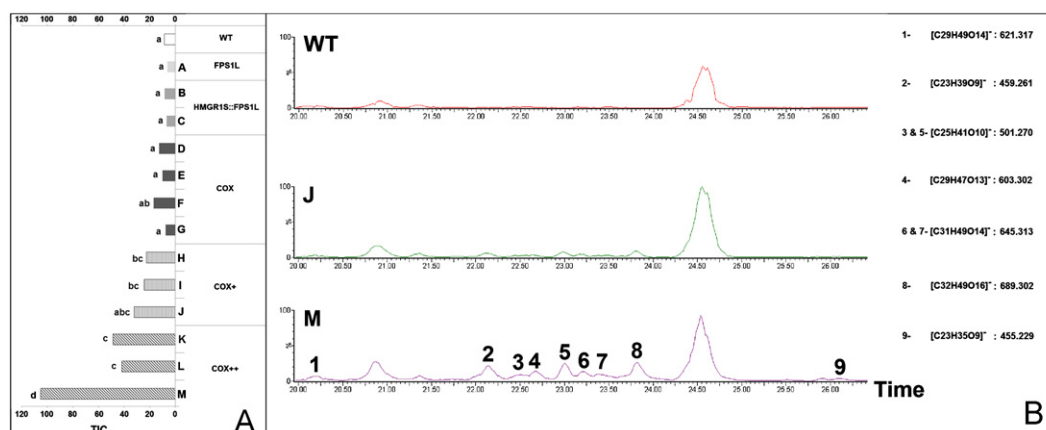


Fig. 3. (A) Accumulated total ion count (TIC) of the LC-QTOF-MS metabolites that correlated with the abundance of linalool and (E)-nerolidol in the transgenic lines. Different letters on the left side of the bars indicate a significant difference ($\alpha=0.05$). (B) Representative chromatograms of a wild type (WT), COX+ (line J) and COX++ (line M) plant in the retention interval (~6 min) where all of the non-volatile metabolites correlating with linalool and nerolidol (indicated by numbers) were detected. Chromatograms were scaled to the highest TIC. The corresponding elemental composition proposed for the parental negative ions of the metabolites are shown. COX, CoxIV-FaNES1 lines; COX+, FPS1L::CoxIV-FaNES1 lines and COX++, HMGR1S::FPS1L::CoxIV-FaNES1 lines (CoxIV, the mitochondrial signal).

non-volatile metabolites a high correlation with the emission of linalool and/or (E)-nerolidol was demonstrated in the RDA plot (Fig. 2D). ANOVA confirmed this correlation as the accumulated total

ion count of all the RDA pinpointed non-volatile metabolites were significantly different between transgenic lines ($\alpha=0.05$) (Fig. 3A). These metabolites resulted in a short interval in the LC-QTOF-MS

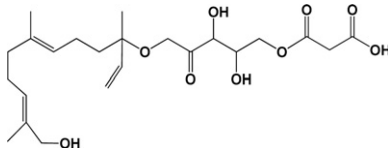
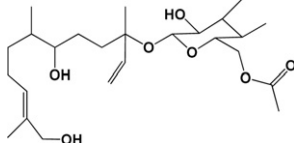
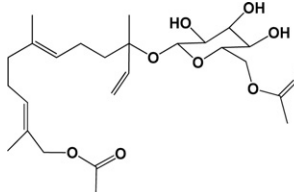
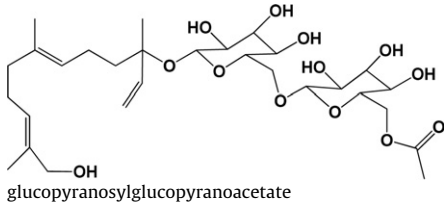
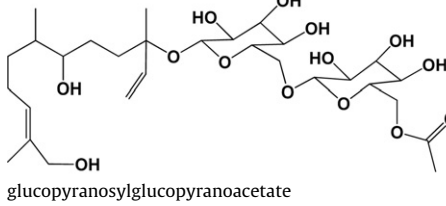
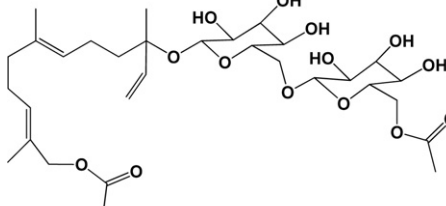
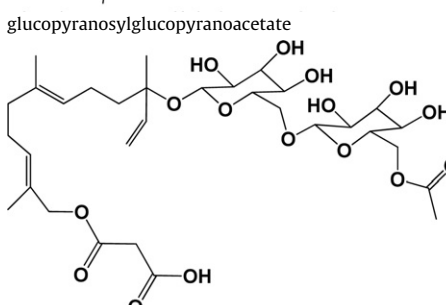
chromatograms, where all these metabolites were present in COX+ and COX++ lines while they were absent in all other transgenic lines and wild type plants (Fig. 3B).

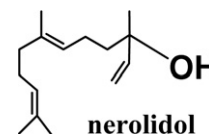
The RDA plot (Fig. 2D) also exposes other volatile and non-volatile metabolites that are correlating with each other. As the

representing arrows are pointing in another direction (vertical), they do not correlate with the abundance of the transgenic volatiles. Hence, they were not selected for further investigations.

In-silico identification using the *Seven Golden Rules* software (Kind and Fiehn, 2007) revealed the most likely elemental

Table 2
Putative annotation of the proposed elemental compositions for the selected parental ion masses in LC-QTOF-MS. Proposed structures are based on the most common structures of naturally occurring terpenes glycosides. A molecule of nerolidol is shown in the first row right.

Negative ion mass	Proposed elemental composition	Putative annotation
455.229	[C ₂₃ H ₃₅ O ₉] [−]	 hydroxynерolidol-malonyl-ketopentoside
459.261	[C ₂₃ H ₃₉ O ₉] [−]	 dihydroxy-hydronerolidol-glucopyranoacetate
501.270	[C ₂₅ H ₄₁ O ₁₀] [−]	 hydroxynерolidol-acetyl-glucopyranoacetate
603.302	[C ₂₉ H ₄₇ O ₁₃] [−]	 hydroxynерolidol-glucopyranosylglucopyranoacetate
621.317	[C ₂₉ H ₄₉ O ₁₄] [−]	 dihydroxy-hydronerolidol-glucopyranosylglucopyranoacetate
645.313	[C ₃₁ H ₄₉ O ₁₄] [−]	 hydroxynерolidol-acetyl-glucopyranosylglucopyranoacetate
689.302	[C ₃₂ H ₄₉ O ₁₆] [−]	 hydroxynерolidol-malonyl-glucopyranosylglucopyranoacetate



composition for the parental ion mass corresponding to the non-volatile metabolites with high correlation to linalool and (*E*)-nerolidol in the RDA plot. In this way all selected metabolites could putatively be annotated as derivatives of (*E*)-nerolidol including glycosylated (with hexose or pentose), acetylated and malonylated derivatives of hydroxynornerolidol (Table 2). A structure was also proposed for the annotated metabolites based on the most common structures of previously reported terpene conjugates (Aharoni et al., 2003; Yang et al., 2011) (Table 2). Following this approach we could not find any linalool derivative.

3.5. Behavioural assays using parasitoid wasps

The effect of the introduced volatile compounds on the behaviour of parasitoid wasps was investigated in a closed Y-tube olfactometer using lines I and L, representative for COX+ and COX++ groups, respectively. Plants of these two lines equally overexpressed *FPS1L* and *HMGR1S* (Supplementary Fig. 1), evenly expressed the *FaNES1* transgene (data not shown), consistently produced (*E*)-nerolidol and more linalool than the wild type plants with the COX++ producing significantly more than COX+ in the second headspace experiment (Fig. 1). The attraction of naive female *D. semiclausum* parasitoid wasps to the volatile blend of non-infested transgenic lines was compared with that of the blend of wild type *A. thaliana* plants. To investigate the potential changes in plant-parasitoid interaction upon infestation, we conducted similar assays in parallel using *P. rapae*-infested transgenic and wild type plants.

The effect of (*E*)-nerolidol on the attraction of parasitoid wasps was examined by using pure (*E*)-nerolidol; 69.0% of *D. semiclausum* wasps significantly preferred the (*E*)-nerolidol containing air over the air without (*E*)-nerolidol (chi-square test, $p < 0.01$, $n = 42$) (Fig. 4).

D. semiclausum parasitoid wasps significantly preferred the blend of volatiles emitted by non-infested COX+ transgenic plants to those of non-infested wild-type plants as 66.4% of responsive wasps chose the blend of non-infested COX+ transgenic plants (chi-square test, $p < 0.01$, $n = 131$, Fig. 4). Similarly, the blend of non-infested COX++ plants was significantly more attractive for parasitoid wasps than that of non-infested wild-type plants, attracting 64.2% of the responsive wasps (chi-square test, $p < 0.01$, $n = 134$) (Fig. 4).

Herbivory by *P. rapae* caterpillars resulted in a higher attractiveness for parasitoid wasps as the blend of wild-type infested plants attracted 75% of the wasps in comparison to the blend of

non-infested wild-type plants (chi-square test, $p < 0.01$, $n = 40$) (Fig. 4). When transgenic plants were infested by caterpillars, the behaviour of the parasitoids did not change in comparison with non-infested plants. Infested COX+ plants attracted 64.8% of the wasps (chi-square test, $p < 0.05$, $n = 71$) and infested COX++ plants attracted 60.7% of wasps (chi-square test, $p < 0.05$, $n = 89$) when tested against infested wild-type (Fig. 4).

4. Discussion

In a previous study, *A. thaliana* Columbia (Col-0) lines expressing mitochondrial targeted *FaNES1* emitted (*E*)-nerolidol and (*E*)-DMNT in their headspace (Kappers et al., 2005). This correlated with enhanced attraction of predatory mites and was proposed as a potential trait for biological control of pests in the field. However, transgenic lines emitted varying amounts of the introduced volatiles and showed retard growth compared with the wild type counterpart. These drawbacks were speculated to be due to the channelling of the mitochondrial FPP towards the biosynthesis of (*E*)-nerolidol by expression of *FaNES1*, decreasing the FPP pool for the other branches of the pathway that are vital for plant growth. The growth retardation would present a barrier for the potential application of volatile compounds pathway engineering for plant defence. In order to reduce the undesired effects, we co-expressed *FPS1L* and *HMGR1S* to improve the flux through the mevalonate pathway.

Headspace analysis of individual plants showed that introduction of *FPS1L* in COX+ and COX++ lines made them more likely to emit (*E*)-nerolidol compared to COX lines. Having larger numbers of emitting plants made COX+ and COX++ lines produce significantly more (*E*)-nerolidol and also linalool in the pooled headspace analysis. These observations suggest that a minimum of precursor or its biosynthesis activity is required for (*E*)-nerolidol biosynthesis through constitutive expression of mitochondrial *FaNES1*.

Furthermore, overexpression of *HMGR1S* also significantly enhanced the production of (*E*)-nerolidol compared with overexpression of *FaNES1* and *FPS1L* alone in COX+ (Fig. 1, experiment 1). However, the amount of the produced (*E*)-nerolidol by individual emitting plants in the COX++ lines does not significantly surpass the level produced by individual emitting plants in COX+ and COX group (Fig. 1, experiment 2). Possibly, the capacity for (*E*)-nerolidol emission is limiting and additional (*E*)-nerolidol is modified and/or conjugated. Indeed, there is a continuous increase in hydroxynornerolidol conjugates in COX, COX+ and COX++ plants.

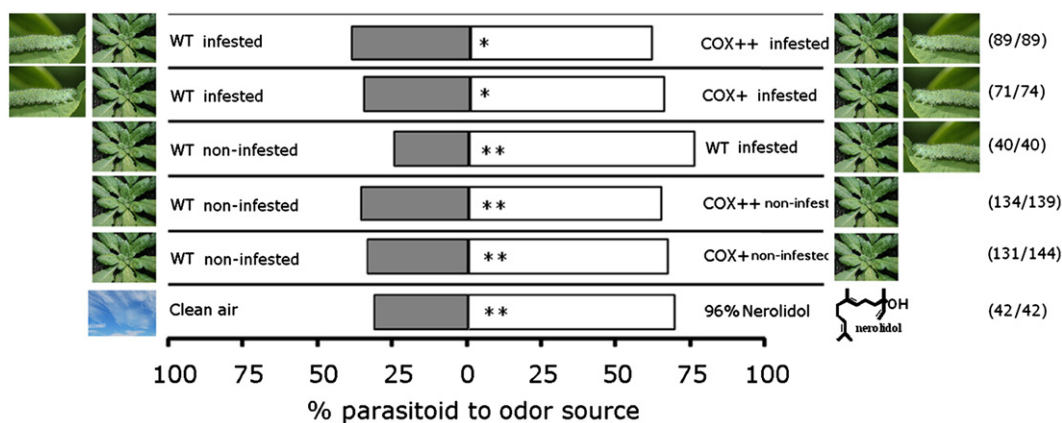


Fig. 4. Responses of naive *D. semiclausum* females to the volatiles of two sets of odor sources, as assessed in a Y-tube olfactometer. Each bar represents the percentage of choices for each of the two odor sources. The number of responding wasps of the total number of tested wasps is given between parentheses. Asterisks indicate significance within a choice test; * $p < 0.05$ and ** $p < 0.01$ (Chi-square test). COX+, *FPS1L*::CoxIV-*FaNES1* lines and COX++, *HMGR1S*::*FPS1L*::CoxIV-*FaNES1* lines (CoxIV, the mitochondrial signal).

In our LC–QTOF–MS analysis no linalool conjugates were detected. Linalool conjugates were detected in plastidic *FaNES1* expressing *A. thaliana* by enzymatic hydrolysis (glycosidase) and subsequent GC–MS analysis of the released aglycones (Aharoni et al., 2003). In this indirect method of detection, the nature of the original conjugations (glycones) and the hydrolysis effectiveness of all types of glycosides remained unknown. To know the metabolic fate of both linalool and (*E*)-nerolidol, we used an untargeted LC–MS approach, data integration and putative identification by the Seven Golden Rules software to detect novel non-volatile compounds in the transgenic lines. Only (*E*)-nerolidol and no linalool conjugates were detected.

Physiologically, conjugation by glycosides diminishes the chemical reactivity of compounds (Von Rad et al., 2001) and together with malonylation facilitates accumulation, storage or transport of phytotoxic secondary metabolites (Von Rad et al., 2001) to reduce toxicity or store them temporarily for subsequent biotic-induced enzymatic release (Yang et al., 2011), which many result in attraction of beneficial insects (James, 2005) or repellence (Halbert et al., 2009; Wei et al., 2004). Here, we show formation of hydroxynornerolidol conjugates in COX+ and COX++ lines, suggesting phytotoxic effects of this compound in high concentrations. The fact that conjugation of sesquiterpenoid volatiles has not commonly been investigated resulted in underestimation of the functional efficiency of the introduced sesquiterpene synthases (Kappers et al., 2005; Wu et al., 2006; Zhang et al., 2011).

Mitochondria are known sites for FPP biosynthesis (Cunillera et al., 1997) and no monoterpene synthase activity has been reported in these compartments (Yu and Utsumi, 2009). Moreover, the CoxIV signalling peptide is highly efficient in targeting proteins to mitochondria (Köhler et al., 1997) in such levels that no trace of linalool was reported in *CoxIV-FaNES1* lines in a previous study (Kappers et al., 2005). Emission of linalool in wild type plants is limited to the inflorescence stage (Aharoni et al., 2003). Hence, the presence of linalool in vegetative stages of transgenic *FaNES1* lines was not expected. However, both (*E*)-nerolidol and linalool were detected in the headspace of COX, COX+ and COX++ plants in the vegetative stage. The dual monoterpene and sesquiterpene synthase activity of the *FaNES1* recombinant protein allows the production of linalool in the presence of GPP in *E. coli* (Aharoni et al., 2004). Moreover, transgenic plants with plastidial *FaNES1* emitted high levels of linalool and (very) low levels of (*E*)-nerolidol with a prominent retarded growth phenotype (Aharoni et al., 2003). As a result of the dual functionality of the *FaNES1*-encoded protein, we could simultaneously show the possibility of forming comparable and correlated amounts of both types of terpenoids strongly suggesting availability of GPP, the precursor for linalool in the mitochondria. *FPS1L* and its homolog *FPS2* can actually catalyse the two sequential condensation reactions: IPP with first DMAPP to form GPP and GPP with the second IPP leading to FPP formation (Cunillera et al., 1996; Delourme et al., 1994). The results show that GPP apparently available allowing *de novo* linalool biosynthesis in substantial amounts, even if *FPS1L* is not over-expressed (in COX lines). The relatively high positive correlation ($R=0.67$) between (*E*)-nerolidol and linalool emission supports the assumption that both products are formed by *FaNES1*. Nevertheless, this speculation needs to be supported by experimental evidence and does not exclude the possibility of the exchange of prenyl precursors including GPP between the plastids, cytosol and the mitochondria, as demonstrated by several authors for plastids and cytosol (Adam and Zapp, 1998; Lichtenthaler, 2000). Wu et al. (2006) targeted limonene synthase to the cytosol and reported low but measurable levels of the monoterpene limonene, indicative of a small cytosolic pool of GPP. Therefore, leakage of the mitochondrial targeted *FaNES1* to the cytosol can be also an

explanation for the presence of linalool despite the high efficiency of CoxIV signalling peptide (Köhler et al., 1997).

Our data do not show any association between biosynthesis of *FaNES1* transgene products and growth retardation, as most of the transgenic lines did not significantly differ from the wild type counterpart or even appeared significantly larger in size. A smaller plant size was also observed within the lines in the *HMGR1S::FPS1L* group without any trace of (*E*)-nerolidol in their headspace. Our observations suggest that the retarded growth phenotype can be due to the constitutive expression of introduced transgenes by using the CaMV 35S promoter and/or positional effect of the transgenes. However, these speculations do not exclude the additive effect of *FaNES1* transgene product toxicity yet such as inhibitory effects of linalool on the respiratory chain in mitochondria (Usta et al., 2009) and antioxidants against reactive oxygen species (Pompella et al., 2003) that already has been reported. Whether (*E*)-nerolidol has similar effects on the physiological state of the cell needs to be investigated.

Inconsistent emission of (*E*)-DMNT was observed by the analysed transgenic lines with Col-3 background, despite its more consistent emission in a previous study using *COX-FaNES1* expressing Columbia 0 lines (Kappers et al., 2005). Although the different Columbia accessions are genetically very similar, Col-3 has been described to show several polymorphisms and the genetic background could therefore be of influence in the emission of volatiles. Furthermore, we can relate these differences to the fact that in the latter study, SPME GC–MS was used to analyse volatile compounds of the detached leaves. (*E*)-DMNT is an inducible volatile (Lee et al., 2010) and possibly the conversion of (*E*)-nerolidol to (*E*)-DMNT in the study by Kappers et al. (2005) was enhanced because leaves were detached for the SPME GC–MS analysis.

D. semiclausum wasps significantly prefer the volatile blend of transgenic COX+ and COX++ plants over that of the wild type. They are also more attracted to the volatile source when it either contains pure (*E*)-nerolidol (versus clean air) or the headspace blend of induced plants (versus non-induced). Wild type Columbia vegetative plants are known to lack linalool (Aharoni et al., 2003). Although linalool has been reported to be attractive to predatory mites (Dicke et al., 1990), transgenic linalool producing *Arabidopsis* plants repel aphids (Aharoni et al., 2003) and *D. semiclausum* wasps are not attracted to linalool as well (unpublished data). Even though multivariate data analysis does not show any other major difference between the headspace of non-infested wild type and transgenic plants other than (*E*)-nerolidol and linalool, it seems that the presence of linalool has no effect on the attraction of wasps in the presence of (*E*)-nerolidol. Interestingly, the attraction of *D. semiclausum* is not disrupted when a non-host caterpillar, *P. rapae*, damages the transgenic lines and induces specific herbivory induced volatiles.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2012.10.002>.

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