Research Article

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Genetic engineering of plant volatile terpenoids: effects on a herbivore, a predator and a parasitoid

Martine Kos,[a]* Benyamin Houshyani,[b] Aart-Jan Overeem,[a] Harro J Bouwmeester,[b] Berhane T Weldegergis,[a] Joop JA van Loon,[a] Marcel Dicke[a] and Louise EM Vet[a,c]

Abstract

BACKGROUND: Most insect-resistant transgenic crops employ toxins to control pests. A novel approach is to enhance the effectiveness of natural enemies by genetic engineering of the biosynthesis of volatile organic compounds (VOCs). Before the commercialisation of such transgenic plants can be pursued, detailed fundamental studies of their effects on herbivores and their natural enemies are necessary. The linalool/nerolidol synthase gene *FaNES1* was constitutively expressed from strawberry in three *Arabidopsis thaliana* accessions, and the behaviour of the aphid *Brevicoryne brassicae* L., the parasitoid *Diaeretiella rapae* McIntosh and the predator *Episyrphus balteatus* de Geer was studied.

RESULTS: Transgenic *FaNES1*-expressing plants emitted (*E*)-nerolidol and larger amounts of (*E*)-DMNT and linalool. *Brevicoryne brassicae* was repelled by the transgenic lines of two of the accessions, whereas its performance was not affected. *Diaeretiella rapae* preferred aphid-infested transgenic plants over aphid-infested wild-type plants for two of the accessions. In contrast, female *E. balteatus* predators did not differentiate between aphid-infested transgenic or wild-type plants.

CONCLUSION: The results indicate that the genetic engineering of plants to modify their emission of VOCs holds considerable promise for facilitating biological control of herbivores. Validation for crop plants is a necessary next step to assess the usefulness of modified volatile emission in integrated pest management.

Supporting information may be found in the online version of this article.

Keywords: *Arabidopsis thaliana*; cabbage aphid; genetic engineering; natural enemy; terpenoid; transgenic plant

1 INTRODUCTION

Over the last decades, the demand for environmentally friendly pest control in agriculture has increased. To meet this demand, integrated pest management (IPM) has been developed as an approach that integrates, for instance, breeding for host plant resistance and biological and cultural control, including a chemical control component only as a last resort.[1–3] Recently, it has been suggested that insect-resistant transgenic crops may become vital components of IPM.[4,5] At present, all commercially available insect-resistant transgenic crops employ direct resistance, and the majority of these transgenic crops express genes coding for proteins naturally occurring in *Bacillus thuringiensis* (*Bt*) that are lethal for target herbivores[6,7] In contrast, indirect resistance, which influences the effectiveness of natural enemies of herbivores,[8,9] has largely been neglected as a trait amenable to transgenesis.

Herbivore damage induces the emission of volatile organic compounds (VOCs) that have been shown to attract natural enemies of herbivores.[8,10–12] Transgenic plants that enhance the effectiveness of natural enemies can be developed by using genetic engineering of VOC biosynthetic pathways.[13–16] There are several benefits of incorporating transgenic crops that enhance biological control into IPM. For example, pest resistance to a biological control agent is not likely to evolve,[17] and the VOCs that transgenic plants emit can repel herbivores.[18–21] However, there may also be ecological risks involved in using these transgenic plants, because the modified emission of VOCs might not only repel certain herbivores, it might also attract others.[22,23] Therefore, before transgenic crops with enhanced indirect resistance are implemented, information on the effects of the modification on (potential) pest herbivores is necessary.

* *Correspondence to: Martine Kos, Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), PO Box 50, 6700 AB Wageningen, The Netherlands. E-mail: M.Kos@nioo.knaw.nl*

a *Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands*

b *Laboratory of Plant Physiology, Wageningen University, Wageningen, The Netherlands*

c *Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands*

Transgenic crops exhibiting enhanced attraction of natural enemies by the emission of novel VOCs or enhanced emission of native VOCs are not yet commercially available. Before commercialisation of these transgenic plants can be pursued, detailed fundamental studies of their effects on different groups of herbivores and their natural enemies are necessary. In the last decade, a few laboratory and field studies have been performed, mostly with the model plant *Arabidopsis thaliana*. These studies show that the novel or enhanced emission of VOCs by genetic engineering can increase the attraction of predatory mites,[24] parasitoid wasps[25,26] and entomopathogenic nematodes,[14,27] and can repel aphids[28] and moths.[21] However, other functional groups of natural enemies that are important for biological control of pest herbivores, such as predacious hoverflies, have not been tested yet. Furthermore, comprehensive studies that simultaneously test the effects of transgenic plants with modified VOC emission on different functional groups of natural enemies and their host or prey species are needed.

The objectives of this study were to test the effects of transgenic *A. thaliana* plants with modified VOC emission on the behaviour of the specialist cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae), the aphid parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae) and the aphid predator *Episyrphus balteatus* de Geer (Diptera: Syrphidae). The model plant *A. thaliana* is ideally suited for testing effects of genetic engineering because it is easily transformed and has a short life cycle.[28] The linalool/nerolidol synthase gene *FaNES1* was constitutively expressed from strawberry in *A. thaliana* and the enzyme was targeted to the mitochondria, which has been shown to result in the emission of two common herbivore-induced plant volatiles: the sesquiterpene (*E*)-nerolidol and its derivative, the homoterpene (*E*)-DMNT (4,8-dimethylnona-l,3,7-triene).[24] Furthermore, because FaNES1 is a dual-function enzyme that catalyses the formation of both nerolidol from FDP (farnesyl diphosphate) and linalool from GDP (geranyl diphosphate) with equal efficiency,[29] the introduction of this enzyme in *A. thaliana* can also lead to emission of the monoterpene linalool if GDP is present.[28] The modified VOC emission by transgenic *FaNES1*-expressing plants resulted in the attraction of predatory mites to these transgenic plants,[24] but other natural enemies have so far not been studied. In the literature, the effects of the manipulation of terpenoid biosynthesis in *A. thaliana* on insects are studied almost exclusively in the accession Col-0.[21,24,28] The present study employed the novel approach of using different *A. thaliana* accessions as background for the transformation and testing the effects of the transformation on the emission of transgene-related products and subsequently on the behavioural response of aphids, parasitoids and predators. Furthermore, the method of Kappers *et al.*[24] was modified by adding the endogenous gene farnesyl diphosphate synthase (*FPS2*) to the *FaNES1* construct to ensure that plants produced enough precursor for (*E*)-nerolidol biosynthesis.

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

Three *Arabidopsis thaliana* (L.) Heynh. accessions were selected that differ in their volatile blend:[30,31] Antwerpen (An)-1 [obtained from the European Arabidopsis Stock Centre (http://nasc.nott.ac.uk/); An-1 = N944], Columbia (Col)-0 (provided by Dr P Reymond, Lausanne, Switzerland); Eringsboda (Eri) (collected in Sweden by members of the Laboratory of Genetics, Wageningen University; Eri-1 = CS22548).

Arabidopsis thaliana seeds were surface sterilised overnight by a vapour-phase sterilisation method. The seeds were placed in a desiccator containing a mixture of 3 mL of hydrochloric acid (37%; Merck KGaA, Darmstadt, Germany) and 100 mL of bleach. The seeds were then inoculated on MS medium (purified agar 0.8% + 2.2 g l^{-1} 0.5 MS + vitamins; pH 6; 35 µg ml^{-1} kanamycin was added to the medium used for the T2 transgenic lines). After 4 days of stratification at 4 °C, plates were transferred to a growth chamber at 21 ± 2 °C, 50–70% relative humidity (RH) and a 8 : 16 light : dark (L : D) photoregime [with a light intensity of 200 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD)].

Two-week-old seedlings with two true leaves were transplanted to pots (5 cm diameter) containing autoclaved soil (80 °C for 4 h; Lentse Potgrond, Lent, The Netherlands). Plants were watered 3 times a week, and the soil was treated weekly with entomopathogenic nematodes (*Steinernema feltiae*; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) to control infestation by larvae of sciarid flies. Plants used in the experiments were 6–7 weeks old and remained in the vegetative state during the experiments.

2.2 Transgenic *A. thaliana* plants

The strawberry linalool/nerolidol synthase gene *FaNES1* and the endogenous gene *FPS2* were constitutively expressed using the 35S CaMV promotor, and the enzymes were targeted to the mitochondria of *A. thaliana*. *FPS2* was overexpressed to ensure that plants produced enough precursor for (*E*)-nerolidol biosynthesis. The supporting information (Methods S1) provides a detailed description of the generation of transgenic *FaNES1*-expressing plants. T1-generation seedlings were selected on kanamycin plates (purified agar 0.8% + 2.2 g l^{-1} 0.5 MS + vitamins; pH 6; 50 µg ml^{-1} kanamycin), and the effectiveness of transformation was confirmed by gene-specific PCR. T2-generation seedlings were also selected on kanamycin plates, and seedlings of one line per accession that were able to grow (hereafter named An-FaNES1, Col-FaNES1 and Eri-FaNES1) were used in the experiments.

2.3 Insect rearing

Brevicoryne brassicae was reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) in a greenhouse compartment.

Diaeretiella rapae was reared in gauze cages (30 × 40 × 60 cm) containing *B. brassicae*-infested *B. oleracea* plants in a climate chamber. Wasps were provided with water and honey. The *B. brassicae* and *D. rapae* cultures originated from individuals collected on *B. oleracea* in the vicinity of Wageningen (The Netherlands) in 2009.

Episyrphus balteatus pupae were provided by Koppert Biological Systems and kept in gauze cages (67 × 50 × 67 cm) in a greenhouse compartment. Adults emerging from the pupae were provided with water, a *B. brassicae*-infested plant, organic sugar grains and bee-collected pollen provided by Koppert Biological Systems. All insect species were reared at 22 ± 2 °C, 60–70% RH and a 16 : 8 h L : D photoregime.

2.4 Chemical and morphological plant traits

2.4.1 Plant morphology

Of ten plants per *A. thaliana* line, foliar biomass (fresh weight), plant diameter and the number of leaves were measured, and trichome density was quantified by counting the number of trichomes in a 25 mm^2 area in the central part of the abaxial side of one mature

leaf on a stereomicroscope (Leitz Dialux 20 EB; Leitz, Wetzlar, Germany; magnification 40×).

2.4.2 *Dynamic headspace collection of aphid-infested plants*

During testing of the preference of *D. rapae* in a Y-tube olfactometer (see below), the headspace of the plants was collected simultaneously. Volatiles were collected by sucking air out of each cuvette at a rate of 90 mL min^{-1} for 3 h through a stainless steel cartridge (Markes, Llantrisant, UK) filled with 200 mg of Tenax TA (20/35 mesh; Grace-Alltech, Deerfield, IL). The fresh weight of the plant foliage in each cuvette was measured after volatile collection. For each *A. thaliana* line, four to six replicates, each consisting of the headspace of four plants, were collected. Volatiles from empty cuvettes were collected to correct for background volatiles.

The headspace samples were analysed using a Thermo Trace GC Ultra (Thermo Fisher Scientific, Waltham, MA) coupled to a Thermo Trace DSQ (Thermo Fisher Scientific, Waltham, MA) quadrupole mass spectrometer (MS) (see Methods S2 in the supporting information for a detailed description of the analytical method and the identification of the volatile compounds). The peak areas of all compounds were expressed per unit plant fresh weight. In the GC-MS and statistical analysis, the emission of the transgene-related products (*E*)-nerolidol, (*E*)-DMNT and linalool was focused upon in particular.

2.5 Aphid performance

Tests were conducted to establish whether aphid performance differed between the transgenic and wild-type line of each accession, because this might (partly) explain the behaviour of the aphid's natural enemies. Aphid performance was assessed in a climate chamber at 21 ± 2 °C, 50–70% RH and a 8 : 16 L : D photoregime. Individual plants with insects were confined to cylindrical plastic containers (height 13 cm, diameter 11 cm) with a gauze lid. The light intensity at plant level was 200 µmol m^{-2} s^{-1} PPFD. Plants were watered once a week. Several six-week-old plants of each accession/line were inoculated with ten adult aphids. After 24 h, the adult aphids were removed, and the produced offspring were allowed to develop for 3 days until they reached the second instar (L2). Three L2 nymphs were transferred to each of 25 plants of the same line as that on which these nymphs had been feeding before. Daily, survival of the nymphs was recorded per plant until the first aphid on that plant reached the adult stage. The fastest-developing adult was kept on the plant, while the other adults were removed. Any alate (winged) adults (ca 5% of all adults) were excluded from the experiment. The development time until first reproduction (T_d) of the remaining adult was recorded, and the adult fresh weight was measured on a microbalance (Sartorius CP2P, Göttingen, Germany). The adult was allowed to feed on the plant and produce offspring, and, after a certain number of days (equivalent to T_d), the number of offspring (N) produced by the adult was counted. The estimated intrinsic rate of increase (r_m) was calculated for each aphid using the formula: $r_m = 0.738 \times (\ln N)/T_d$.[32]

2.6 Aphid preference

The preference of *B. brassicae* for the transgenic or the wild-type line of each accession was tested in two-choice bioassays in a greenhouse compartment (22 ± 2 °C, 60–70% RH and a 16 : 8 h L : D photoregime). One transgenic and one wild-type plant were connected by a paper bridge (2 × 3 cm). Ten *B. brassicae* adults from the stock rearing were released in the centre of the bridge and were allowed to walk towards and feed on the plants. After 24 h, the number of aphids on both plants was counted. For each accession, at least 22 replicates were performed (An-1: 38; Col-0: 22; Eri: 30).

2.7 Parasitoid and predator preference

The preference of parasitoids and predators for volatiles from the transgenic or the wild-type line of each accession was tested in two-choice bioassays. *A. thaliana* plants (6–7 weeks old) were infested with 100 *B. brassicae* nymphs of mixed instars 3 days prior to the bioassays.

2.7.1 *Parasitoid preference for aphid-induced plant volatiles*

Parasitoid behaviour was assessed in a Y-tube olfactometer in a climatised room at 22 ± 2 °C, as described by Bukovinszky *et al*.[33] Compressed air was filtered over charcoal and split into two air streams, each at a flow of 2 L min^{-1}. Each air stream was led through a 5 L glass cuvette that contained four aphid-infested plants of either the transgenic or the wild-type line of an accession. Each air stream was then led into one of the two arms of the Y-tube. The olfactometer was illuminated from above using artificial light at an intensity of 60 µmol m^{-2} s^{-1} PPFD.

Naive, mated, two-day-old *D. rapae* females were allowed to oviposit for 1 h in aphids feeding on either the transgenic or the wild-type line of an accession (equally divided among the tested wasps) to increase their motivation to search for a host. Immediately after the training period, experienced wasps were individually released at the base of the Y-tube using a glass vial, and their preference for one of both odour sources was recorded. A choice was recorded when a wasp crossed a finish line drawn 1 cm before the end of each arm and did not return to the junction within 15 s. Wasps that did not move into one of the arms within 10 min or did not make a final choice within 15 min were considered to be non-responsive and were omitted from the statistical analysis. Seven to nine new sets of transgenic and wild-type plants were used for each accession (An-1: 9; Col-0: 7; Eri: 7). For every new set of plants, 20 wasps were tested. After every ten wasps, the position of the odour sources was exchanged to compensate for any asymmetry in the set-up.

2.7.2 *Predator oviposition preference*

Mated female hoverflies from the stock rearing were used in the behavioural assays when they were 2–3 weeks old. Females were transferred to a plastic cage (30 × 30 × 30 cm) containing one transgenic plant and wild-type plant of the same accession and a 10% sugar solution in the same greenhouse compartment as described in Section 2.6. Females were allowed to oviposit on the plants. After 24 h, the number of eggs deposited on each plant was counted. Females that did not lay any eggs (about 30% of the females) were eliminated from the analysis. For each accession, at least 23 replicates with ovipositing females were obtained (An-1: 29; Col-0: 33; Eri: 23).

2.8 Statistical analysis

For most analyses, the transgenic line was compared with the wild-type line for each accession. Analyses were performed in SPSS for Windows (18th edition; SPSS, Chicago, IL), unless indicated otherwise. If variables were log transformed to obtain normality and equal variance, this is indicated in the relevant table of Section 3. To test the effect of the line (transgenic or

Table 1. Mean (± SE) morphological plant characteristics of wild-type and transgenic *FaNES1*-expressing lines of three *Arabidopsis thaliana* accessions[a]

	A. thaliana line					
Plant characteristic	An-1	An-FaNES1	Col-0	Col-FaNES1	Eri	Eri-FaNES1
Biomass (fresh weight; g)[b]	0.51 ± 0.02	0.63 ± 0.04*	0.49 ± 0.02	0.36 ± 0.01*	0.62 ± 0.05	0.43 ± 0.03*
Diameter (cm)[b]	8.0 ± 0.1	7.6 ± 0.2 ns	8.2 ± 0.1	6.1 ± 0.2*	8.7 ± 0.2	7.2 ± 0.2*
Number of leaves[c]	21.5 ± 0.3	25.8 ± 1.1*	22.1 ± 0.4	26.3 ± 1.6*	26.7 ± 0.9	28.8 ± 2.1 ns
Trichome density[b,d]	8.1 ± 0.6	10.7 ± 0.8*	13.7 ± 0.8	18.5 ± 1.2*	6.4 ± 0.3	7.8 ± 0.6*

[a] Three wild-type *A. thaliana* accessions were used: An-1, Col-0 and Eri. Of each accession, a transgenic *FaNES1*-expressing line was made, named An-FaNES1, Col-FaNES1 and Eri-FaNES1 respectively.
[b] Analysed by Student's *t*-tests.
[c] Analysed by Mann–Whitney *U*-tests.
[d] Trichome density is the number of trichomes per 25 mm^2 leaf area of mature leaves.
* denotes significant difference ($P < 0.05$), and ns denotes no significant difference ($P > 0.05$) between the wild-type and transgenic line for each accession.
$n = 10$.

wild-type) on the measured variables, Student's *t*-tests were used. If assumptions on normality and equal variance were violated, Mann–Whitney *U*-tests were used. Aphid survival was averaged per plant, and differences in aphid survival between the transgenic and wild-type line of each accession were analysed by logistic regression in GenStat (13th edition; VSN International, UK; dispersion estimated). *T*-probabilities were calculated to test pairwise differences between means. Differences in the emission of transgene-related VOCs (a) among the three transgenic lines and (b) among the three wild-type lines were tested with ANOVA and *post hoc* Tukey tests on the log-transformed data.

Aphid and parasitoid preference, measured by the number of aphids or wasps choosing either the transgenic or the wild-type line of each accession, was analysed using a chi-square test, with the null hypothesis that the aphids or wasps did not have a preference for any of the two lines. Effects of parasitoid experience on the preference of the wasps was tested by logistic regression (dispersion estimated) in GenStat. Predator preference, measured by the number of predator eggs on either the transgenic or the wild-type line of each accession, was analysed with Wilcoxon's matched-pair signed-rank tests.

To test whether there were differences in the entire volatile profile between the transgenic line and the wild-type line of each accession, use was made of multivariate discriminant analysis projection to latent structures – discriminant analysis (PLS-DA) in SIMCA-P (12th edition; Umetrics, Umeå, Sweden).[34]

To preprocess data, volatile amounts were log transformed, mean centred and scaled to unit variance.

3 RESULTS

3.1 Plant morphology

Transgenic An-FaNES1 plants had a higher biomass but a similar plant diameter compared with the An-1 wild-type plants, whereas Col-FaNES1 and Eri-FaNES1 plants had a lower biomass and smaller diameter than their respective wild-type plants (Student's *t*-test, $n = 10$, biomass: for An-1, $t = -3.04$, $P = 0.007$; for Col-0, $t = 6.38$, $P < 0.001$; for Eri, $t = 3.49$, $P = 0.003$; diameter: for An-1, $t = 1.40$, $P = 0.179$; for Col-0, $t = 9.24$, $P < 0.001$; for Eri, $t = 5.69$, $P < 0.001$) (Table 1). Plants of all transgenic lines had a larger number of leaves than plants of the wild-type lines, although this was only significant for accessions An-1 and Col-0 (Mann–Whitney *U*-test, $n = 10$: for An-1, $U = 7.50$, $P < 0.001$; for Col-0, $U = 21.00$, $P = 0.029$; for Eri, $U = 44.50$, $P = 0.684$) (Table 1). For all accessions, leaves of transgenic *FaNES1*-expressing plants had a higher trichome density than leaves of wild-type plants (Student's t-test, $n = 10$: for An-1, $t = -2.74$, $P = 0.013$; for Col-0, $t = -3.49$, $P = 0.003$; for Eri, $t = -2.28$, $P = 0.035$) (Table 1).

3.2 Dynamic headspace collection of aphid-infested plants

Transgenic *FaNES1*-expressing lines of the three accessions emitted (*E*)-nerolidol, whereas wild-type plants of the three accessions did not emit this compound (Fig. 1). An-FaNES1 plants emitted larger amounts of (*E*)-nerolidol than Eri-FaNES1 plants (ANOVA, $F_{2,11} = 6.01$, $P = 0.017$).

The transgenic *FaNES1*-expressing lines emitted larger amounts of (*E*)-DMNT than the corresponding wild-type plants, but the difference was only significant for accessions An-1 and Col-0 (Student's *t*-test: for An-1, $t = -3.38$, $P = 0.019$; for Col-0, $t = -4.06$, $P = 0.007$; for Eri, $t = -1.65$, $P = 0.194$) (Fig. 1). While (*E*)-DMNT was detected in all transgenic samples, it was detected in only two of the six wild-type An-1 samples, one of the four wild-type Col-0 samples and two of the four wild-type Eri samples. Col-FaNES1 plants emitted larger amounts of (*E*)-DMNT than Eri-FaNES1 plants (ANOVA, $F_{2,11} = 4.26$, $P = 0.043$). The wild-type lines did not differ in emission of (*E*)-DMNT (ANOVA, $F_{2,11} = 0.295$; $P = 0.750$).

For each accession, the transgenic *FaNES1*-expressing line emitted larger amounts of linalool than the wild-type plants, but the difference was only significant for accession An-1 owing to the high variation in emission of this compound among replicates (Student's *t*-test: for An-1, $t = -3.55$, $P = 0.015$; for Col-0 and Eri, $P > 0.05$) (Fig. 1). In ca 50% of the wild-type samples, linalool was detected (three of the six An-1 samples, two of the four Col-0 samples and three of the four Eri samples), while this compound was detected in all the transgenic samples. The three transgenic lines did not differ in the emission of linalool, and neither did the three wild-type lines (ANOVA, $P > 0.05$ for both analyses).

Apart from the reported differences in the emission of (*E*)-nerolidol, (*E*)-DMNT and linalool, there were no differences in the overall volatile profile between aphid-infested plants of the transgenic and wild-type line of each accession, as analysed by a multivariate discriminant analysis using all other volatile compounds (no significant PLS-DA components could

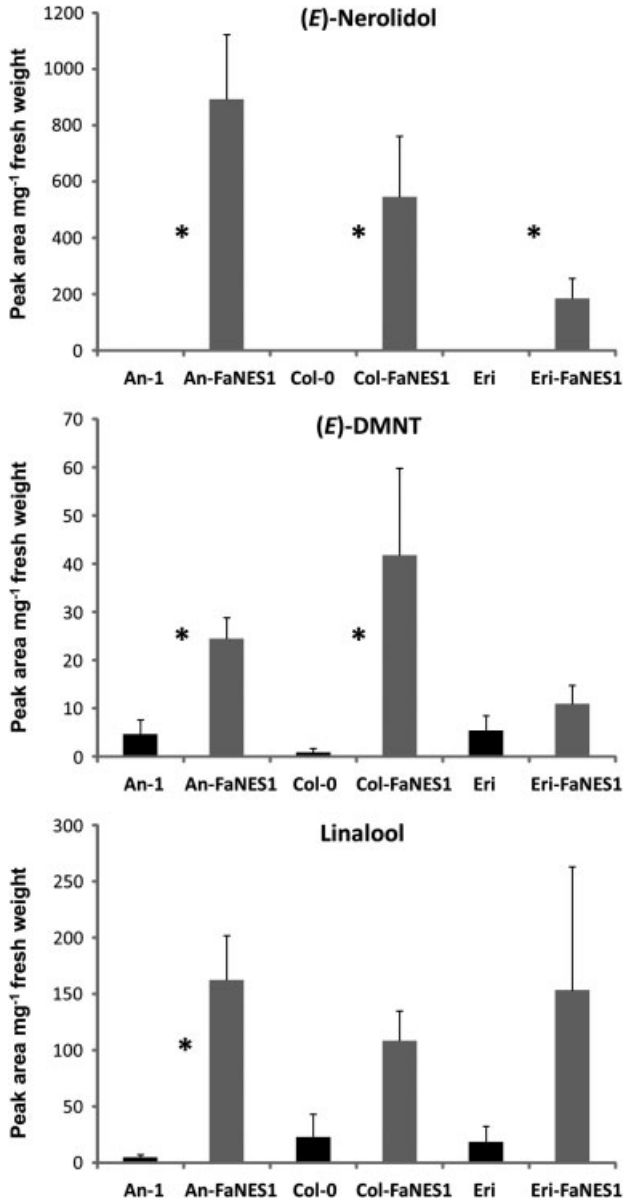

Figure 1. Emission of (*E*)-nerolidol, (*E*)-DMNT and linalool (peak area mg^{-1} fresh weight + SE) by aphid-infested wild-type or transgenic *Arabidopsis thaliana* lines of three accessions (An-1, Col-0 and Eri). Of each accession, a transgenic *FaNES1*-expressing line was made, named An-FaNES1, Col-FaNES1 and Eri-FaNES1 respectively. $n = 4–6$ for each bar. An asterisk indicates a significant difference ($P < 0.05$) in the emission of the compound between the wild-type and the transgenic *FaNES1*-expressing line for each accession, as analysed by Student's *t*-tests.

be extracted; see Table S1 in the supporting information for an overview of the volatile compounds detected in the headspace of the aphid-infested plants).

3.3 Aphid performance

For each of the accessions, *B. brassicae* performance was assessed to investigate whether it differed between the transgenic and wild-type line. There were no differences in any of the aphid performance parameters between transgenic and wild-type plants for each of the accessions ($P > 0.05$ for every comparison) (Table 2).

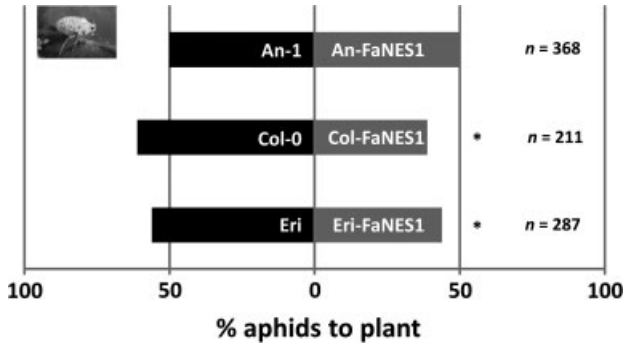

Figure 2. Preference of *Brevicoryne brassicae* adults in two-choice tests for the wild-type or transgenic lines of three *Arabidopsis thaliana* accessions (An-1, Col-0 and Eri). Of each accession, a transgenic *FaNES1*-expressing line with enhanced emission of volatiles was made, named An-FaNES1, Col-FaNES1 and Eri-FaNES1 respectively. Each bar represents the percentage of aphids that made a choice for the indicated plant line. The total number of tested aphids is indicated on the right. An asterisk indicates a significant preference ($P < 0.05$) for one of the two lines in a combination, as analysed by chi-square tests. Photograph by Tibor Bukovinszky; www.bugsinthepicture.com.

3.4 Aphid preference

The preference of *B. brassicae* for the transgenic or the wild-type line of each accession was tested in two-choice bioassays. Aphids preferred wild-type Col-0 and Eri plants over plants of the corresponding transgenic line, but did not differentiate between An-1 and An-FaNES1 plants (chi-square test: for An-1, $\chi^2 < 0.01$, $P = 1.000$; for Col-0, $\chi^2 = 10.47$, $P = 0.001$; for Eri, $\chi^2 = 4.27$, $P = 0.039$) (Fig. 2).

3.5 Parasitoid preference for aphid-induced plant volatiles

Parasitoid preference for the transgenic or the wild-type line of each accession was tested in a Y-tube olfactometer. Female parasitoids preferred volatiles from aphid-infested transgenic An-FaNES1 and Col-FaNES1 lines over volatiles from the corresponding aphid-infested wild-type lines, but did not differentiate between volatiles from aphid-infested Eri and aphid-infested Eri-FaNES1 plants (chi-square test: for An-1, $\chi^2 = 4.12$, $P = 0.042$; for Col-0, $\chi^2 = 5.28$, $P = 0.022$; for Eri, $\chi^2 = 2.84$, $P = 0.092$) (Fig. 3). There was no effect of previous oviposition experience on the preference of the wasps (logistic regression, $P > 0.05$ for every combination).

3.6 Predator oviposition preference

Preference of female *E. balteatus* for the transgenic or the wild-type line of each accession was tested in two-choice bioassays. Females did not differentiate between aphid-infested plants of the transgenic or wild-type line of any of the accessions (Wilcoxon's matched-pair signed-rank test, $P > 0.05$ for every comparison) (Fig. 4).

4 DISCUSSION

4.1 Volatile emission by transgenic plants

Aphid-infested transgenic *FaNES1*-expressing plants emitted the sesquiterpene (*E*)-nerolidol, whereas this compound was not emitted by aphid-infested wild-type plants. Aphid-infested transgenic plants also emitted the homoterpene (*E*)-DMNT. (*E*)-DMNT is synthesised from (*E*)-nerolidol by endogenous *Arabidopsis* enzymes.[24] The emission of (*E*)-nerolidol and (*E*)-DMNT from transgenic *FaNES1*-expressing plants of the accessions An-1, Col-0

Table 2. Mean (± SE) performance characteristics of *Brevicoryne brassicae* on wild-type and transgenic *FaNES1*-expressing lines of three *Arabidopsis thaliana* accessions[a]

	A. thaliana line					
Performance parameter	An-1	An-FaNES1	Col-0	Col-FaNES1	Eri	Eri-FaNES1
Survival until adult stage (%)[b]	87	88 ns	93	89 ns	83	88 ns
Development time until first reproduction T_d (days)[c]	8.8 ± 0.1	8.8 ± 0.1 ns	9.7 ± 0.1	9.8 ± 0.1 ns	9.5 ± 0.1	9.6 ± 0.1 ns
Adult fresh weight (mg)[d]	0.410 ± 0.017	0.417 ± 0.021 ns	0.329 ± 0.015	0.303 ± 0.011 ns	0.339 ± 0.011	0.320 ± 0.011 ns
Number of offspring *N* in time period equivalent to T_d[d]	34.4 ± 3.4	34.0 ± 2.8 ns	24.8 ± 2.5	21.8 ± 2.2 ns	25.4 ± 2.0	23.0 ± 2.9 ns
Estimated intrinsic rate of population increase r_m[c]	0.288 ± 0.007	0.288 ± 0.008 ns	0.233 ± 0.009	0.217 ± 0.010 ns	0.246 ± 0.006	0.230 ± 0.008 ns

[a] Three wild-type *A. thaliana* accessions were used: An-1, Col-0 and Eri. Of each accession, a transgenic *FaNES1*-expressing line was made, named An-FaNES1, Col-FaNES1 and Eri-FaNES1 respectively.
[b] The performance parameter was averaged per plant before statistical analysis and analysed by logistic regression and *post hoc t*-probability tests.
[c] Analysed by Mann–Whitney *U*-tests.
[d] The performance parameter was log-transformed in statistical analysis to obtain normality and analysed by Student's *t*-tests.
ns denotes no significant difference ($P > 0.05$) in performance between aphids feeding on the wild-type and transgenic line for each accession.

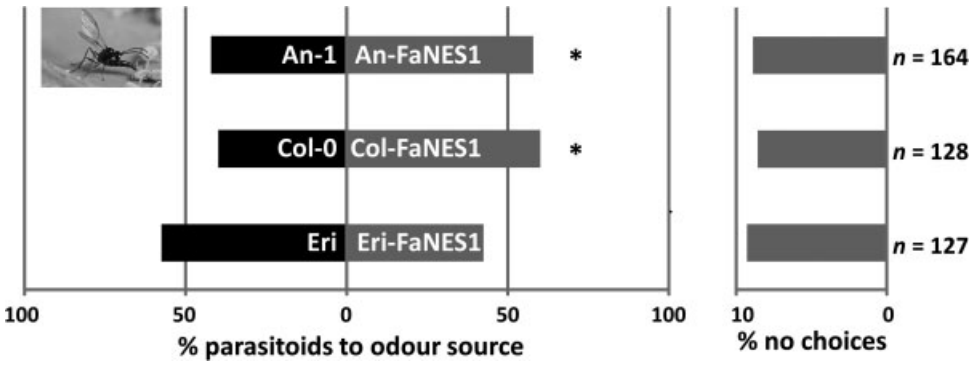

Figure 3. Responses of *Diaeretiella rapae* parasitoid females to volatile blends emitted by aphid-infested wild-type or transgenic *Arabidopsis thaliana* lines of three accessions (An-1, Col-0 and Eri) in a Y-tube olfactometer. Of each accession, a transgenic *FaNES1*-expressing line with enhanced emission of volatiles was made, named An-FaNES1, Col-FaNES1 and Eri-FaNES1 respectively. Each bar represents the percentage of females that made a choice for the indicated odour sources. The percentage of parasitoids that did not make a choice ('% no choices') in each experiment and the total number of tested females are indicated on the right. An asterisk indicates a significant preference ($P < 0.05$) for one of the two lines in a combination, as analysed by chi-square tests. Photograph by Nina Fatouros; www.bugsinthepicture.com.

and Eri is in agreement with the study by Kappers *et al.*[24] on accession Col-0, in which the same *FaNES1* construct was used, but without the *FPS2* construct.

Transgenic *FaNES1*-expressing plants also emitted larger amounts of the monoterpene linalool compared with wild-type plants, although this was only significant for accession An-1. FaNES1 is a dual-function enzyme that catalyses the formation of both linalool from GDP and nerolidol from FDP with equal efficiency.[29] The present finding of higher linalool emission by transgenic plants suggests that there is a small pool of GDP present in the mitochondria of the *A. thaliana* accessions used. However, the finding could also mean that there was leaking of the linalool/nerolidol synthase from the mitochondria of the transformants in the present study, as Kappers *et al.*[24] did not report the emission of linalool in transgenic *FaNES1*-expressing plants.

The detection of (*E*)-DMNT and linalool in some of the wild-type samples is remarkable, as most previous studies report that *A. thaliana* plants do not produce these compounds in the vegetative state.[21,24,28,35] However, these latter studies were done using an older-version mass spectrometer,[35] or used solid-phase microextraction (SPME) GC-MS.[21,24,28] Perhaps their methods were less sensitive for (*E*)-DMNT and linalool than the method used here. In the present study, (*E*)-DMNT and linalool were not detected in all wild-types, suggesting that the amounts were below the detection limit of the GC-MS, and the amounts in the samples in which these compounds were actually detected were small. A more recent study using the same GC-MS set-up also recorded (*E*)-DMNT and linalool [and even (*E*)-nerolidol] in vegetative plants of the three *A. thaliana* accessions studied here,[30] suggesting that *A. thaliana* is indeed able to produce these compounds in the rosette stage.

The present results show that increasing the plant terpenoid emission through genetic engineering is possible not only in *A. thaliana* accession Col-0, which is almost exclusively used in the literature, but also in the other two *A. thaliana* accessions. The level of emission of (*E*)-nerolidol and (*E*)-DMNT by the transgenic plants was dependent on the accession that was transformed. It can be expected that plants with a higher biomass emit larger amounts of volatiles. In agreement with this, An-FaNES1 plants emitted larger amounts of (*E*)-nerolidol than Eri-FaNES1 plants. However, the difference in biomass does not explain why Col-FaNES1 plants emitted larger amounts of (*E*)-DMNT than Eri-FaNES1 plants, as Col-FaNES1 plants had a lower biomass than Eri-FaNES1 plants.

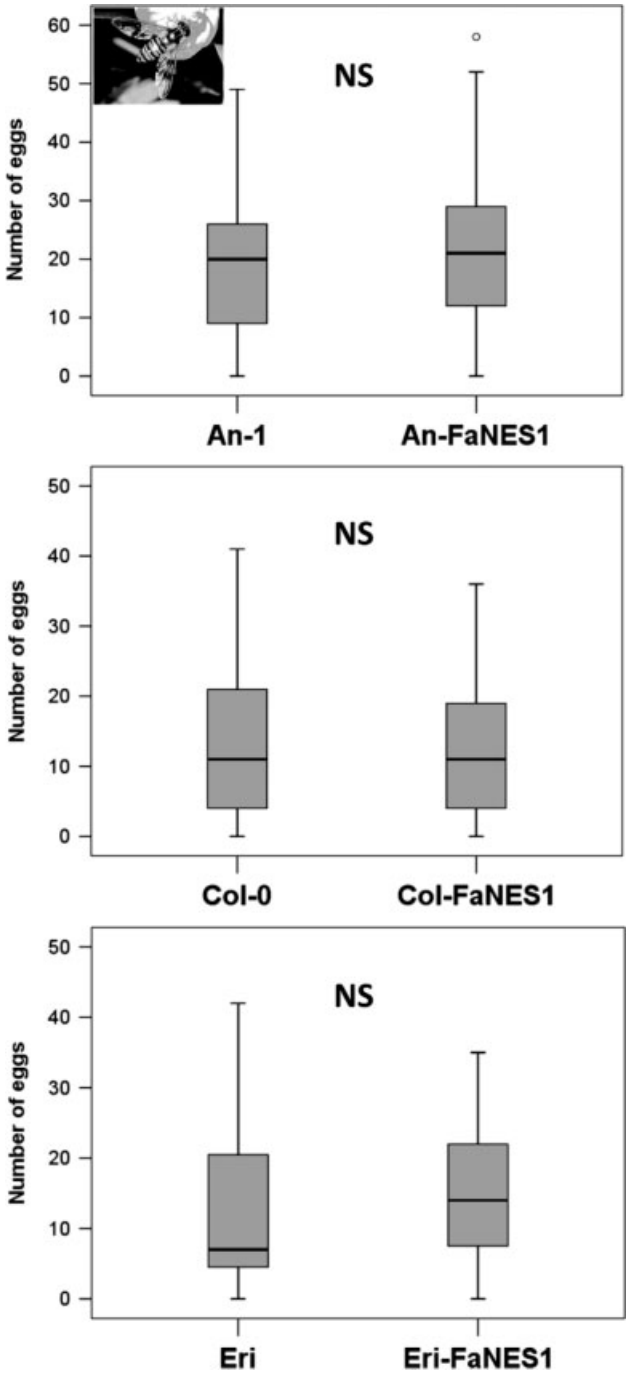

Figure 4. Oviposition preference (number of eggs) of the aphid predator *Episyrphus balteatus* in a two-choice assay with aphid-infested plants of wild-type and transgenic *Arabidopsis thaliana* lines of three accessions (An-1, Col-0 and Eri). Of each accession, a transgenic *FaNES1*-expressing line with enhanced emission of volatiles was made, named An-FaNES1, Col-FaNES1 and Eri-FaNES1 respectively. The boxes span the first to third quartile range, with the line across the box indicating the median. The whiskers represent the range. The open circle in the top part of the figure represents an outlier. NS indicates a non-significant difference ($P > 0.05$) between the number of eggs deposited on the wild-type and the transgenic lines of each accession, as analysed by Wilcoxon's matched-pair signed-rank test. Photograph by Han Endt; www.veluwe-insecten.nl/zweefvliegen/episyrphus/episyrphus.html.

This suggests that the expression of the linalool/nerolidol synthase gene, the efficiency of the produced enzymes or the availability of substrates differs among different accessions. However, because only one transgenic line per accession was studied here, it cannot be excluded that the differences in volatile emission among the transgenic lines resulted from, for instance, the number of gene copies or the position of the inserted gene in the genome, rather than from the genotype that was transformed.

The biosynthesis of the novel terpenoids by transgenic *FaNES1*-expressing plants has been shown to impose costs on plant growth, and was speculated to be due to a reduction in the availability of substrates for other metabolites that play an important role in plant growth, or to direct toxic effects of the transgenic products to the plant.[24,28] In agreement with this, growth retardation of transgenic plants was observed for two of the three accessions (Col-0 and Eri). However, An-FaNES1 plants, which had the highest emission of (*E*)-nerolidol and linalool in their headspace, were actually larger than the wild-type An-1 plants. This suggests that the effects on plant growth are dependent on the genetic background of the transgenic line, or on the exact insertion position of the transgene in the genome.

Transgenic *FaNES1*-expressing plants of each accession exhibited a higher trichome density than the corresponding wild-type plants. This is probably due to the transgenic plants having a smaller diameter than the wild-type plants, suggesting that the leaves of the transgenic plants were smaller. It is known that trichomes are produced at the beginning of the development of a leaf, and the growth of new trichomes is limited when the leaf starts to expand.[36]

4.2 Aphid performance and preference

The performance of the aphid *B. brassicae* did not differ between transgenic and wild-type plants for any of the three accessions. This suggests that the transgenic and wild-type plants did not differ in quality for aphid development, even though the transgenic plants had higher trichome densities, and were smaller (for two of the three accessions). Transgenic *FaNES1*-expressing plants that emitted large amounts of linalool (because the enzyme was targeted to the plastids instead of the mitochondria) also did not affect the performance of caterpillars of the herbivore *Plutella xylostella*.[21]

Brevicoryne brassicae aphids preferred wild-type Col-0 and Eri plants over plants of the corresponding transgenic line, suggesting that the volatiles (*E*)-nerolidol, (*E*)-DMNT and/or linalool emitted by Col-FaNES1 and Eri-FaNES1 plants acted as repellents to *B. brassicae*. This is in agreement with the study by Aharoni *et al.*,[28] in which transgenic *FaNES1*-expressing plants with a high emission of linalool and linalool derivatives and a low emission of nerolidol repelled the aphid *Myzus persicae*. Linalool and/or nerolidol were also found to be repellent to spider mites, thrips and moths.[21,37–40] However, repellence of linalool and/or nerolidol to *B. brassicae* does not explain why aphids did not differentiate between An-1 and An-FaNES1 plants, the latter producing the largest amounts of (*E*)-nerolidol and linalool of the three transgenic lines. Effects of plant biomass or trichome density or of an interaction between these variables and VOC emission on aphid preference cannot be ruled out. Unfortunately, it was not possible to test the preference of *B. brassicae* in the Y-tube olfactometer, because the aphids did not respond to any odour source in this set-up (personal observation).

4.3 Parasitoid preference

The aphid parasitoid *D. rapae* preferred plants of An-FaNES1 and Col-FaNES1 over the wild-type plants. *Diaeretiella rapae* parasitises many aphid species on at least 16 plant species,[41] which could explain why this parasitoid is attracted towards plants emitting (*E*)-nerolidol, (*E*)-DMNT and linalool, all three common herbivore-induced volatiles. (*E*)-nerolidol, the most dominant transgene-related product in the headspace of the transgenic plants, is often reported as a component of the volatile blend of herbivore-induced plants. However, to the present authors' knowledge, attraction of natural enemies of herbivores specifically towards a source emitting this compound in pure form has only been reported for predatory mites.[24] The attractiveness of (*E*)-DMNT and linalool to several species of natural enemies has often been reported.[24,42–47] Parasitoids did not differentiate between Eri and Eri-FaNES1 plants, probably because Eri-FaNES1 plants emitted only small amounts of (*E*)-nerolidol and (*E*)-DMNT.

4.4 Predator preference

In contrast to the parasitoid wasp, females of the aphid predator *E. balteatus* did not differentiate between aphid-infested plants of the transgenic and wild-type line of each accession. This suggests that (*E*)-nerolidol, (*E*)-DMNT and linalool do not affect the behaviour of *E. balteatus* at the emission rates tested. *Episyrphus balteatus* mainly uses aphid-derived chemicals to locate its prey.[48] There are unfortunately only a few published studies on the role of volatiles in hoverfly attraction. Verheggen *et al*.[49] recorded that the sesquiterpenes α-humulene and β-caryophyllene did not evoke electroantennographic (EAG) responses in *E. balteatus*, whereas the aphid alarm pheromone (*E*)-β-farnesene did evoke responses. Monoterpenes elicited only a low response, compared with the response to green leaf volatiles, and adding the monoterpene limonene to uninfested plants did not affect the oviposition behaviour of females.[49] The low response to plant terpenoids might explain why *E. balteatus* did not differentiate between transgenic plants and wild-type plants in the present study, which differed only in emission of three terpenoids.

In contrast to *D. rapae*, during the behavioural bioassays, *E. balteatus* had access to the aphid-infested plants and could therefore also have used plant cues other than volatiles, as well as aphid cues, to select a plant for oviposition. Hoverflies were not observed to prefer the wild-type plants, which had lower trichome densities, over the transgenic plants, although it has been shown before that female hoverflies have difficulties in landing on plants with high trichome densities.[50] Aphid body weight and population size did not differ between transgenic and wild-type plants, which may explain why hoverflies did not differentiate between the plants.

4.5 Potential of application of the transgenic approach in IPM

The present results indicate that genetically engineered plants with modified emission of VOCs can enhance the attraction of natural enemies of herbivores. Furthermore, a potential pest herbivore was repelled by the transgenic plant, while its performance was not affected, which increases the potential of these transgenic plants for pest control. However, effects on other pest insects need to be taken into account, and field trials are necessary to validate the results obtained in this study. Although the present study suggests that genetically engineering plants to modify their emission of VOCs holds considerable promise for improving control of herbivores, it was performed with the model plant *A. thaliana*. The results have to be validated with actual crop species before such a transgenic approach can be applied in IPM. The recent study by Degenhardt *et al*.[27] is an excellent example of how this technique could be successfully applied in a crop plant and under field conditions.

Before implementation of transgenic plants with modified emission of VOCs in IPM can be pursued, the technique for creating these transgenic plants should be optimised. Similarly to previous studies,[24,27,28] a constitutive promotor was used, which results in the constitutive production of the transgene-related products. Replacement of the constitutive promotor with an inducible promotor, for example one that is induced by herbivore feeding, will be essential. Natural enemies are capable of learning, and, if the VOCs are emitted constitutively, the natural enemies likely learn to ignore the signal if it is not associated with a host or prey.[4,51] Furthermore, by using an inducible promotor, negative effects of the constitutive production of the transgenic products on plant growth may be prevented.[21] IPM involves a combination of pest management techniques, including breeding for resistance. Such resistance does not have to be absolute, but may be partial, and breeding for crops that enhance the effectiveness of biological control agents through genetic modification is a new development.[4] Crops that enhance the effectiveness of biological control agents and repel pests are likely to be a novel component of future IPM.[4,27]

4.6 Conclusion

The present study shows that genetically transforming plants to modify the emission of terpenoids can repel a herbivore and enhance the attraction of one of its natural enemies, holding promise for improving control of herbivores in agriculture. However, the results have to be validated for a crop species and under field conditions before transgenic plants with enhanced attraction of natural enemies can provide interesting new components for IPM.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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