Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior

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The alarm pheromone for many species of aphids, which causes dispersion in response to attack by predators or parasitoids, consists of the sesquiterpene (*E*)-β-farnesene (*E*βf). We used high levels of expression in *Arabidopsis thaliana* plants of an *E*βf synthase gene cloned from *Mentha* × *piperita* to cause emission of pure *E*βf. These plants elicited potent effects on behavior of the aphid *Myzus persicae* (alarm and repellent responses) and its parasitoid *Diaeretiella rapae* (an arrestant response). Here, we report the transformation of a plant to produce an insect pheromone and demonstrate that the resulting emission affects behavioral responses at two trophic levels.

Diaeretiella rapae | *Myzus persicae* | semiochemical | volatile | farnesene

Aphids (Homoptera: Aphididae) in the subfamily Aphidinae include some of the world's major insect pests. When attacked by predators or parasitoids, aphids produce a sticky defensive secretion from two organs (the siphunculi) on their dorsal surface which interferes with feeding or oviposition by the attacker. At the same time, many aphids release an alarm pheromone that for many species consists of the sesquiterpene hydrocarbon (*E*)-β-farnesene (*E*βf), which causes other aphids in the vicinity to stop feeding and to move away (1). *E*βf can also increase the proportion of aphid progeny that are alate (winged) (2). Furthermore, it functions as a kairomone in attracting predators (3) and enhances the foraging behavior of parasitoids (4–6). The potential for *E*βf in the control of pest aphids has long been recognized (7) and was demonstrated by showing that increased mobility of aphids, caused by application of synthetic *E*βf, could improve the uptake of contact pesticides (8) or transmission of biological control agents (9). However, the use of *E*βf in novel plant protection strategies has been hampered by its chemical instability and high volatility, despite improvements offered by the synthesis of propheromones (10), analogues (11), and derivatives (12), and the exploitation of natural sources (13). In the wild potato *Solanum berthaultii* (Solanaceae), release of endogenous *E*βf from specialized foliar trichomes causes repulsion of aphids (14). This fact suggests that an effective way to employ *E*βf in plant protection would be to modify crop plants genetically to produce *E*βf (15) as an aphid repellent. However, many plants producing *E*βf naturally also emit a wide range of other sesquiterpenes, beyond the traces of *E*βf isomers produced by some aphids (16), particularly β-caryophyllene and (−)-germacrene D, which are inhibitory to the alarm activity (17, 13). This finding demonstrates the adaptation of aphids to discriminate between the extremely pure *E*βf produced by conspecifics and the mixture of related compounds produced naturally by plants. Indeed, in *S. berthaultii*, the pure *E*βf produced in the foliar trichomes, without the background of inhibitory compounds released by the leaf, enables a strong repellent effect to occur (14). Any attempt to engineer plants to resist aphids by release of *E*βf, therefore, needs to ensure that the *E*βf is of high purity and produced in sufficient quantity to swamp other endogenous plant sesquiterpenes, thereby eliciting aphid host avoidance behavior. Emission of *E*βf would also be expected to cause increased foraging by predators and aphid parasitoids.

The work presented here differs from two recently published articles in which *Arabidopsis thaliana* was genetically engineered to produce new plant volatiles by using plant sesquiterpene synthases (18, 19). Thus, a nerolidol synthase was expressed in *A. thaliana*, and the high expression of nerolidol, achieved by targeting the subcellular location of the synthase to the mitochondria, allowed generation in the plant of an addition product of nerolidol that was responsible for preference by predatory spider mites (18). Also in *A. thaliana*, expression of a maize terpene synthase gene (*tps10*) known to be responsible for making a specific blend of sesquiterpenes attractive to wasps parasitizing maize herbivores caused expression of the same blend of sesquiterpenes, thereby rendering the transgenic *A. thaliana* attractive to the natural enemies of maize pests (19). In both of these cases, introduction of a single gene stimulated the indirect defense of host plants by attracting predators or parasitoids to the plant-derived signals. Here we show that, in addition to affecting tritrophic interactions by making the transgenic plants more attractive to parasitoids, the behavior of the pest is, itself, modified. Although aphids can also synthesize *E*βf, we have used a plant *E*βf synthase to produce a volatile normally produced by the aphid pest.

Results and Discussion

A. thaliana is a useful experimental plant with which to investigate heterologous expression of sesquiterpene synthase. It has a relatively low level of endogenous terpene biosynthesis, and much is already known about its molecular genetics and terpene emission profile (20–24). A gene encoding an *E*βf synthase has been cloned from *Mentha* × *piperita* and functionally expressed in *Escherichia coli* (25, 26). In the latter study (26), we found the *in vitro* product profile of the synthase to consist of essentially pure *E*βf with no other detectable sesquiterpenes, and this gene was therefore considered to be ideal for engineering production of the aphid alarm pheromone into plants. In the research reported here, we transferred the *E*βf synthase cDNA (26) into *A. thaliana* (Col-0) under the control of a constitutive cauliflower mosaic virus 35S promoter. The volatiles from flowering wild-type and transgenic plants were isolated by air entrainment from intact plants and analyzed by coupled GC-MS (27). This analysis showed that wild-type plants produce predominantly β-caryophyllene, together with small amounts of other sesquiterpenes known from this source (20, 22, 23) (Fig. 1*A*). In comparison, transgenic lines showed a large peak from *E*βf (Fig. 1*B*), with very small amounts of other sesquiterpenes such as

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Abbreviation: *E*βf, (*E*)-β-farnesene.

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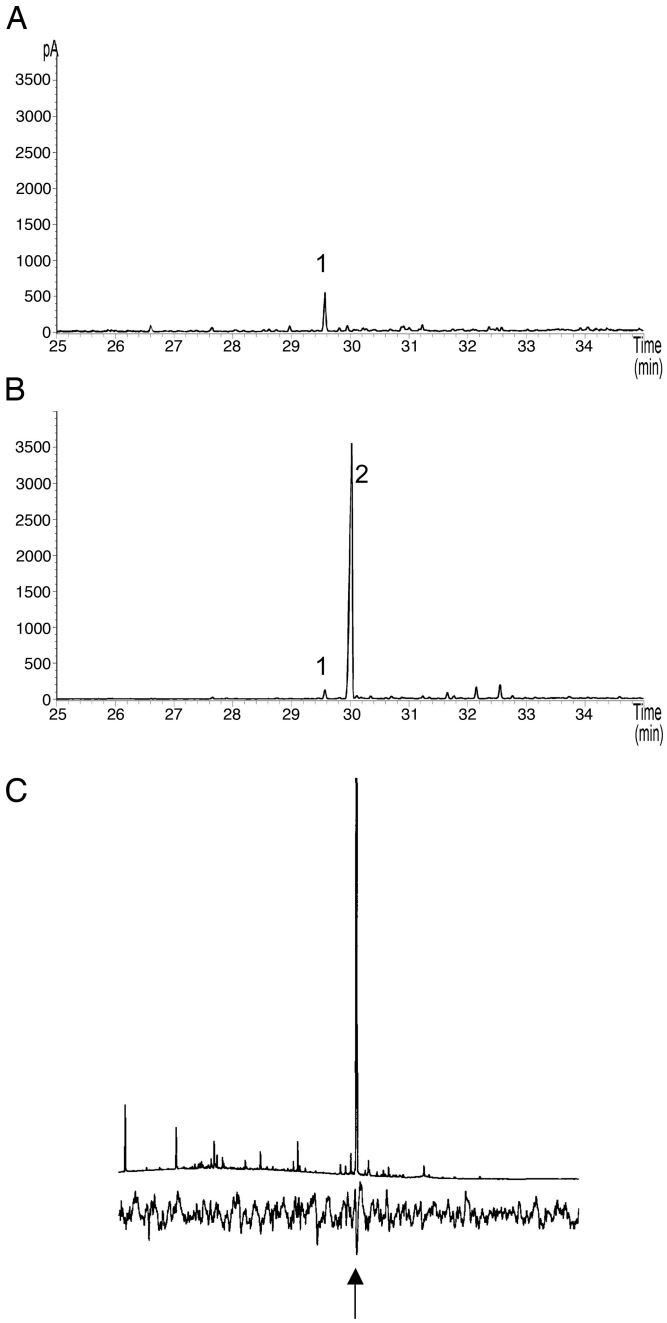

Fig. 1. Sesquiterpene region of the gas chromatograms (HP-1 column, flame ionization detector) of the volatile chemicals collected from flowering *A. thaliana* (Col-0) wild-type (*A*) and the transgenic line FS11-4 (*B*). β-Caryophyllene (peak 1) is the main constituent of the headspace of the wild-type plant, whereas *E*βf (peak 2) is the major component of the transgenic plant volatiles, which also contain a greatly reduced amount of β-caryophyllene. (*C*) Coupled GC–electroantennography using antennae from a female aphid parasitoid, *D. rapae*. The effluent from the GC column was split and directed simultaneously to the GC detector and the antennal preparation. The upper trace is the GC of air entrainment volatiles from transgenic line FS9-2; the lower trace shows the electroantennograph response (marked by an arrow) to the major peak, *E*βf.

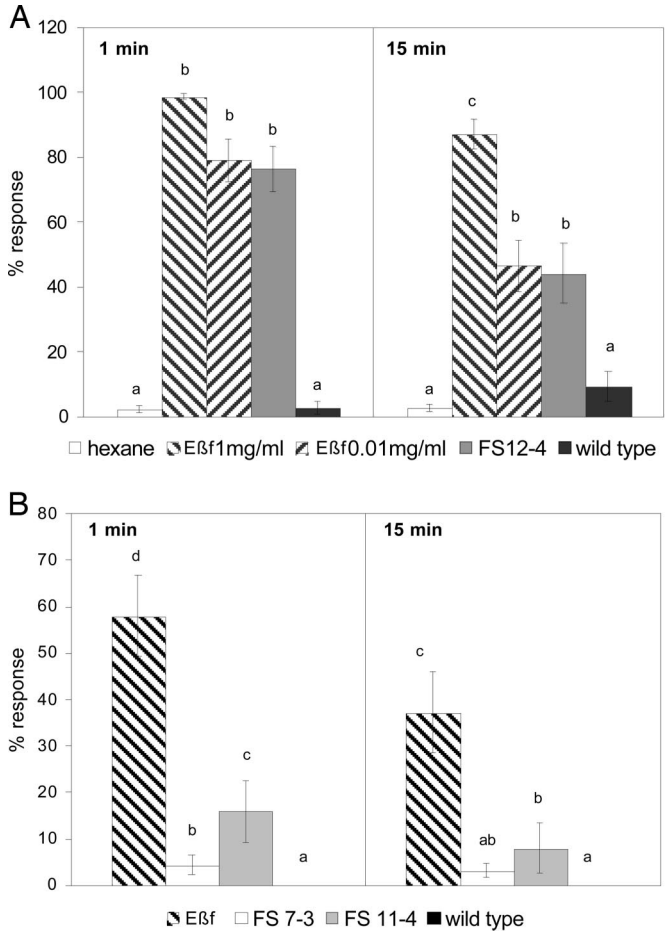

Fig. 2. Alarm response of colonies of *M. persicae* to volatiles released by *A. thaliana* (Col-0). The percentage of aphids moving after 1 min and 15 min was recorded, and the arcsin-transformed data were subjected to ANOVA. Columns with different letters indicate responses that are significantly different ($P < 0.05$; $n = 10$). (*A*) Response to 1-μl droplets of air entrainment samples of transgenic and wild-type *A. thaliana* volatiles, compared with the response to synthetic *E*βf in hexane and a hexane control. (*B*) Response to 20-ml samples of vapor from the headspace above individual plants of transgenic and wild-type *A. thaliana*, enclosed in 2-liter vessels, and synthetic *E*βf.

caryophyllene. Levels of *E*βf produced by individual transformed plants ranged from undetectable to >880 ng/h. Four transgenic lines were selected for insect behavioral experiments: FS7-3, FS9-2, FS11-4, and FS12-4. Because only limited numbers of plants from each line were produced, not all lines could be tested in every bioassay. However, the volatiles produced by these four lines, analyzed by GC, showed qualitatively similar terpenoid emission profiles (emission of *E*βf ranged from 384 to >880 ng/h at the flowering growth stage). *E*βf emission was found to vary more with the age of the plant than among the four lines. Wild-type plants did not produce detectable levels of *E*βf. No phenotypic differences were seen between wild-type and transgenic lines, as judged by general growth rate, size of the plant, or time of flowering (28), suggesting that synthesis of *E*βf had little or no effect on general metabolism, in contrast to the changes in metabolism found by overexpression of nerolidol synthase (21). In one experiment, the production of *E*βf from a typical flowering transgenic plant (FS9-2) was 483 ng/h, and that of other sesquiterpenes was 67 ng/h, whereas for the wild-type flowering plant, total sesquiterpene production was 110 ng/h (3-h collection). Thus, the production of *E*βf appears to be at the expense of other sesquiterpenes, presumably by competing for the common substrate, farnesyl diphosphate.

*E*βf was also detected in plants at the rosette growth stage. From a typical transgenic plant without flowers (FS12-4), *E*βf production was 205 ng/h, and production of other sesquiterpenes was 26.7 ng/h, whereas for the wild-type, total sesquiter-

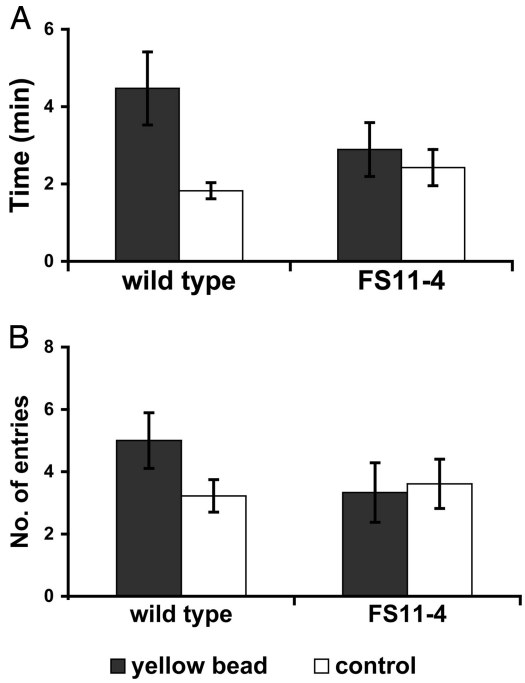

Fig. 3. Responses of *M. persicae* to an attractive visual cue (a yellow bead) placed in the treated arm of a four-arm olfactometer, in combination with volatiles from wild-type and transgenic *A. thaliana* FS11-4. Time spent (*A*) and number of entries (*B*) into treated and control arms are compared. Mean values ($n = 6$) are shown; bars indicate ± SE. The yellow bead was significantly attractive to aphids when presented together with wild-type volatiles ($P = 0.021$ for time spent; $P = 0.034$ for number of entries; paired *t* test). However, with volatiles from the transgenic *A. thaliana* line FS11-4, the yellow bead was no longer attractive.

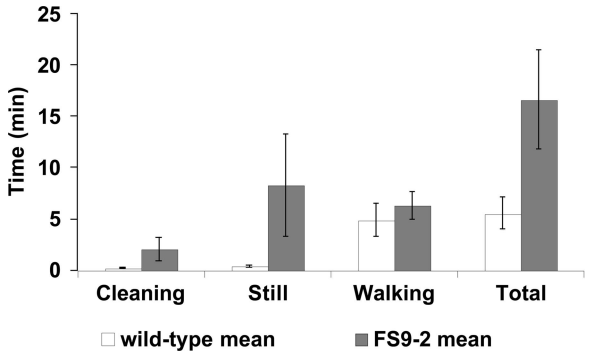

Fig. 4. Time spent by foraging *D. rapae* on wild-type and transgenic *A. thaliana* plants. Parasitoids were released at the center of the plants, and individual behaviors were scored by using THE OBSERVER software. Mean values ($n = 10$) are shown; bars indicate ± SE. The total amount of time spent foraging was significantly higher on the transgenic *A. thaliana* line FS9-2 ($P = 0.026$, unpaired *t* test).

pene production was 7.1 ng/h (21-h collection). Total sesquiterpene synthase activity in leaves of the transgenic line FS9-2 was 2,000 ± 320 ng/g of fresh weight per hour, compared with 330 ± 50 ng/g of fresh weight per hour in wild-type leaves. Inasmuch as the strong constitutive 35S promoter expresses transgenes in most plant parts, our data confirm that endogenous synthesis of farnesyl diphosphate is highest in the flowers, which are the major site of sesquiterpene production in wild-type plants. In the leaves of transgenic plants, the large relative increase in $E\beta$f emission suggests that endogenous sesquiterpene emission in these organs is not specifically limited by supply of substrate but may be transcriptionally regulated.

Several transgenic lines of *A. thaliana* expressing $E\beta$f were tested for their effect on the aphid *Myzus persicae* by using three methods: (*i*) exposure to a droplet of hexane containing entrained volatiles from a transgenic plant or pure synthetic $E\beta$f, (*ii*) exposure to air from the headspace above *A. thaliana* plants at the flowering growth stage, and (*iii*) a four-arm olfactometer that measured diversion from the visual attraction of a yellow bead (13). In *i* (Fig. 2*A*), the aphid response to volatiles from a transgenic line (FS12-4), producing 880 ng/h $E\beta$f, was comparable to the response observed with a 0.01 mg/ml solution of synthetic $E\beta$f, both after 1 min and after 15-min exposure, and was significantly higher than the response to wild-type volatiles or the solvent control. There was a greater response to 1 mg/ml $E\beta$f, but this response was significant only after 15 min. In *ii* (Fig. 2*B*), aphid response to vapor from two transgenic plants (FS7-3 and FS11-4, producing 822 and 802 ng/h $E\beta$f, respectively), was significantly greater after 1 min of exposure than the response to the wild-type ($P < 0.01$). The third test (*iii*) (Fig. 3) showed that normal attraction to a yellow bead was rendered ineffective by the repellency of plant-produced $E\beta$f. Thus, transgenic plants of the type produced in this study would be expected to have a reduced level of attack by aphids. Although plant repellency could provide a component of integrated control for pest aphids, it would need to deter all aphids because, when a plant is colonized by even a single individual, parthenogenetic reproduction will rapidly lead to a large population.

Manipulation of parasitic wasps (parasitoids) presents an alternative, or at least an additional, approach to aphid control (29). Because $E\beta$f is known to act as a kairomone in attracting aphid parasitoids (4, 5), the effect of transgenic plants on the foraging behavior of *Diaeretiella rapae* (Hymenoptera: Braconidae) (6) was also tested. *D. rapae* is a parasitoid specialized for aphids feeding on members of the Brassicaceae and hence is appropriate for use with *A. thaliana.* We first confirmed that antennae of *D. rapae* were physiologically able to respond to the $E\beta$f produced by transgenic *A. thaliana* plants (Fig. 1*C*). The behavioral response was then investigated by releasing individual *D. rapae* directly onto plants and recording the time spent before the parasitoid flew away (30, 31). Our results showed a highly significant increase in time spent by foraging parasitoids on the transgenic *A. thaliana* plants (Fig. 4). Most of this increase was in the time spent remaining still on the plant, suggesting an arrestment or "sit and wait" response in the absence of aphid prey. The practical implication of this finding, and the influence of learning behavior, remain to be investigated in field simulation studies.

The current research shows production of an insect pheromone in a transgenic plant and clearly indicates the potential in aphid control for plants expressing an $E\beta$f synthase gene. This approach is particularly appropriate for improving the level of aphid control achieved by aphid parasitoids. The successful use of an $E\beta$f synthase gene is in marked contrast to similar attempts to produce germacrene A in transgenic plants (21). However, in two recent studies, sesquiterpene synthases have been expressed in *A. thaliana* (18, 19), and it was demonstrated that the emitted volatiles from these enzymes were able to influence parasitoid behavior. The applied value of the current work will need to be determined by experiments with transgenic crop plants such as canola or oilseed rape (*Brassica napus*). These transgenic plants can also be produced by using promoter sequences that facilitate the specific induction of $E\beta$f production after exposure to natural plant activators (32), at times when aphids are expected to attack. Redirection of gene expression to increase substrate availability may also be worthwhile (18, 33).

Materials and Methods

Transgenic *A. thaliana*. *A. thaliana* (Col-0) plants were transformed, by the floral dip method (33), with the construct p35S::*E*βfS and the *E*βf synthase fused to the cauliflower mosaic virus 35S promoter. This gene was isolated from *Mentha* × *piperita* and functionally characterized in *E. coli* (26) before use in the construct. To identify transgenics, T_1 seeds from these plants were germinated on selection plates containing the antibiotic kanamycin. Healthy seedlings were assumed to be transgenic and were transferred to soil to continue growth. The presence of the transgene was confirmed by PCR, and up to 60 T_1 plants were allowed to self-fertilize and to set seed. Homozygous plants from lines showing a high level of expression of *E*βf were generated for the behavioral work with aphids.

To determine whether transgenic lines containing the p35S::*E*βfS construct were expressing the transgene, the headspace above the flowers of wild-type *A. thaliana* and *E*βf lines was sampled. GC analysis (e.g., Fig. 1) of transgenic lines, including FS11-4, showed the presence of a large hydrocarbon peak, which was determined to be *E*βf by comparison of mass spectra and coinjection with the authentic standard on two columns of different polarity (for general GC and GC-MS conditions, see ref. 1). Levels produced by individual plants ranged from undetectable to >880 ng/h of sampling. Significant levels of *E*βf were also found in the headspace above young leaf material. To screen a larger number of lines, a simple enzyme assay was conducted with tritiated farnesyl pyrophosphate substrate to determine the total sesquiterpene synthase activity in plant tissue extracts. All plants, irrespective of the level of production of *E*βf, had normal growth and development.

Air Entrainment and GC. The volatile chemicals from the headspaces of wild-type and transgenic plants were collected by entrainment onto Tenax TA resin (60/80 mesh, 0.05 g; Supelco) contained in a glass GC inlet liner between glass-wool plugs. Plants were placed singly in a sealed 1-liter glass chamber, and charcoal-filtered air was admitted at the bottom of the vessel. The Tenax tube was inserted in the top of the chamber, and headspace air was drawn through the tube at a rate of 750 ml/min for periods ranging from 1 to 21 h.

The collected volatiles were analyzed by GC (Agilent, Edinburgh, U.K.) on a nonpolar column (HP-1, 50 m × 0.32 mm i.d. × 0.52-μm film thickness) with detection by flame ionization. The volatiles were transferred onto the column by inserting the Tenax tube into a programmable temperature vaporization inlet (programmable injector; Anatune, Cambridge, U.K.) programmed to heat from 30°C to 220°C in 12 sec.

Electrophysiology. Electroantennogram recordings were made by using Ag/AgCl glass electrodes filled with saline solution. A female parasitoid, *D. rapae*, was anesthetized by chilling, and the head was excised and placed in the indifferent electrode. The tips of the terminal processes of the antennae were removed to ensure a good contact with the electrolyte in the recording electrode. The signals generated by the antennae were passed through a high-impedance amplifier (UN-06; Syntech, Hilversum, The Netherlands). The coupled GC–electrophysiology system, in which the effluent from the GC column is split and directed simultaneously to the antennal preparation and the GC detector, has been described in ref. 34. The outputs from the electroantennogram amplifier and the GC detector were monitored simultaneously and analyzed by using a customized software package (Syntech).

Behavioral Assays: Aphids. ***Droplet test.*** A colony of 30–50 individuals of *M. persicae*, feeding on uncut leaves of Chinese cabbage (*Brassica rapa* var. *pekinensis*) was placed in a constant flow of air. A 1-μl droplet of hexane, containing either pure synthetic *E*βf (3) of known concentration or volatiles trapped from the headspace above *E*βfS-expressing *A. thaliana* or wild-type plants, was applied to the leaf, and the insect behavior was observed. The numbers of aphids responding were counted after 1 min and again after 15 min.

Headspace test. Colonies of *M. persicae* similarly feeding on Chinese cabbage were tested with 20 ml of vapor containing synthetic *E*βf (5 μg) or with headspace volatiles collected from individual transgenic plants or from wild-type *A. thaliana* enclosed in a 2-liter vessel. The number of aphids showing a response was recorded as above.

Olfactometer bioassay. Individual alate *M. persicae* were introduced into a four-arm olfactometer (13). A yellow bead, giving a clear visual cue, was placed in the treated arm and moist filter paper in the remaining control arms. Air from either a transgenic *E*βf-producing line or from wild-type plants was drawn into the treated arm across the yellow bead. The length of time spent and the number of entries into the treated arm were recorded and compared with those for the control arms.

Behavioral Assays: Parasitoid. Our experimental procedure was similar to that described in ref. 31. Plants at growth stage 3.50 (50% of final size) (28) were mounted on a turntable that allowed them to be examined even when the parasitoid moved to the back of the plant. Once an individual parasitoid was released, the time it spent walking, remaining still, or cleaning was recorded. An observation was terminated when the parasitoid flew away from the plant. THE OBSERVER software (Version 4.1; Noldus Information Technology, Wageningen, The Netherlands) was used for recording the behavioral observations.

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