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Attractiveness of Constitutive and Herbivore-Induced Sesquiterpene Blends of Maize to the Parasitic Wasp *Cotesia marginiventris* (Cresson)

Anna Fontana · Matthias Held · Chalie A. Fantaye · Ted C. Turlings · Jörg Degenhardt · Jonathan Gershenzon

Abstract Plant volatile compounds induced by herbivore attack have been demonstrated to provide a signal to herbivore enemies such as parasitic wasps that use these volatiles to locate their hosts. However, in addition to herbivore-induced volatiles, plants often release volatiles constitutively. We assessed the interaction between herbivore-induced and constitutively released volatiles of maize in the attraction of the wasp *Cotesia marginiventris* that parasitizes herbivorous lepidopteran larvae feeding on maize. Experiments were carried out with olfactometers in which the sources of volatiles were transgenic *Arabidopsis thaliana* plants overexpressing maize sesquiterpene synthases that produce blends of herbivore-induced or constitutive compounds. We found that the constitutive volatiles of maize terpene synthase 8 (TPS8) were attractive to *C. marginiventris*, just like the herbivore-induced volatiles of TPS10 studied earlier. A mixture of both the TPS8 and TPS10 volatile blends, however, was more effective in parasitoid attraction, indicating that constitutively released sesquiterpenes enhance the attraction of those induced by herbivores. While *C. marginiventris* did not distinguish among the volatiles of TPS8, TPS10, nor those of another maize sesquiterpene synthase (TPS5), when these blends were combined, their attractiveness to the wasp appeared to increase with the complexity of the blend.

Key Words *Cotesia marginiventris* · *Arabidopsis thaliana* · *Zea mays* · *Spodoptera littoralis* · Sesquiterpenes · Volatile organic compounds (VOCs) · Herbivore-induced volatiles · Constitutive volatiles · Plant defense · Parasitoid attraction

Introduction

Plants emit a plethora of volatile organic compounds (VOCs) from their vegetative organs into the surrounding atmosphere. The most common are green leaf volatiles (GLVs, C_6 alcohols, aldehydes, and derivatives), and terpenes, especially monoterpenes and sesquiterpenes (Dudareva et al., 2004). VOCs are released abundantly after herbivore attack, but undamaged plants, especially tree species, also show high emission rates (Kesselmeier and Staudt, 1999). For example, undamaged poplars (*Populus* spp.) can invest more than 10% of their photosynthetically fixed carbon into the emission of isoprene (Brilli et al., 2009). The majority of studies on vegetative VOCs have focused on herbivore-induced volatiles and provided ample evidence for their role

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in attraction of herbivore enemies. These studies include agronomically important crops (e.g., maize, tomato, soybean, cabbage) as well as wild species, both herbaceous and woody (reviewed by Unsicker et al., 2009). Much less is known about the role of constitutively emitted volatiles in plant indirect defense, as these rarely have been studied separately from the herbivore-induced ones. Attraction of herbivore enemies to uninfested plants has been documented for some aphid parasitoids (Hymenoptera: Braconidae) (reviewed by Hatano et al., 2008) and for two lepidopteran parasitoids (Elzen et al., 1986, 1987).

Herbivore-induced and constitutive VOCs often are released in complex blends (Dicke et al., 2009). Since these natural mixtures are difficult to manipulate or to reproduce synthetically, most studies have tested arthropod attraction to either whole blends, or to single VOCs available in pure form. For example, the GLV (*E*)-3-hexenol is attractive to the braconid wasp *Apanteles* (=*Cotesia*) *kariyai* (Watanabe) (Takabayashi et al., 1991), and the sesquiterpenes γ-bisabolene and (*E*)-β-caryophyllene attract *C. sonorensis* females. These ichneumonids oviposit in noctuid larvae that feed on cotton plants, whose vegetative parts are rich in sesquiterpenes (reviewed by Rutledge, 1996). Single volatiles responsible for the attraction of herbivore enemies also have been identified in a few aphid-parasitoid systems (reviewed by Hatano et al., 2008) and in one mite - predatory mite system (de Boer and Dicke, 2004; de Boer et al., 2004). However, there are cases in which the whole volatile blend released by a plant is necessary for the parasitoid to locate its host's habitat. A study with the egg parasitoid *Closterocerus ruforum* (Krausse) (Hymenoptera: Eulophidae) demonstrated that the whole VOC blend emitted by Scots pine (*Pinus sylvestris* L.) after oviposition by the herbivorous sawfly *Diprion pini* (L.) attracts the parasitoid (Mumm and Hilker, 2005). This blend differs from that of uninfested plants by having greater amounts of (*E*)-β-farnesene. However, neither (*E*)-β-farnesene, nor the VOC blend of uninfested plants by itself was attractive to the parasitoids; both were required.

Maize (*Zea mays* L.) is one of the best studied species for the role of volatiles in indirect plant defense. The volatile bouquet emitted by seedlings after feeding by generalist lepidopteran caterpillars (*Spodoptera littoralis* (Boisd.), *S. exigua* (Hübn.)) has been well characterized and studied for over two decades (D'Alessandro et al., 2009; Turlings et al., 1990). Important components of this odor are GLVs (esters and aldehydes) with their abundance dependent on the extent and intensity of herbivore damage. The monoterpene alcohol linalool, the C_{11} homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and indole also are present often in high amounts. However, the biggest proportion of the blend is made up of (*E*)-β-farnesene, (*E*)-α-bergamotene, and other sesquiterpene hydrocarbons (Turlings and Ton, 2006). This herbivore-induced blend is attractive to parasitic wasps (Turlings et al., 1990, 1991), raising the question of whether the entire mixture or individual components are responsible for the attraction. In order to tackle this question, the volatile mixture was fractionated (D'Alessandro and Turlings, 2005), and plants have been engineered to emit only a specific fraction of the terpene blend. Transgenic *Arabidopsis thaliana* plants emitting the volatiles formed by maize terpene synthase 10 (TPS10), which make up most of the herbivore-induced sesquiterpenes of maize leaves, were more attractive than untransformed controls to the parasitoid wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) in olfactometer experiments (Schnee et al., 2006).

In addition to herbivore-induced terpenes, many lines of maize emit an additional sesquiterpene mixture, which is constitutively emitted by the roots and aboveground vegetative tissues of young plants. These terpenes are synthesized by TPS8 (Fig. 1a), a terpene synthase that is expressed independently of herbivory in the inbred line B73 as well as in several other cultivars and wild ancestors of maize (Köllner et al., 2004b, 2008, 2009). The ecological role of maize TPS8 products has been investigated in the context of plant-pathogen interactions and deterrence of insect herbivory, and these substances have been shown to act as defenses against fungi, but not against herbivores (Fontana et al. unpublished data). The terpene blend produced by TPS8 is extraordinarily complex,and one major component of this mixture, (*E*)-β-caryophyllene, has been implicated in the attraction of herbivore enemies both above and below ground (Köllner et al., 2008). A third sesquiterpene blend dominated by sesquithujene and β-bisabolene is produced by TPS5 in the husks and at lower levels in the leaves of mature maize plants (Köllner et al., 2004a). As young instars of generalist lepidopteran larvae are not normally found on these plant parts, the TPS5 blend is unlikely to be used by their parasitoids for host location.

Here, we studied the function of the constitutively emitted TPS8 terpenes in indirect plant defense. The study system involved the generalist parasitic wasp *C. marginiventris,* which oviposits on lepidopteran larvae feeding on maize. The constitutively emitted TPS8 volatiles may act: 1) together with the herbivore-induced (TPS10) terpenes to strengthen attraction of the wasps; 2) antagonistically with the herbivore-induced signal to weaken the parasitoid attraction; or 3) without effect, neither enhancing nor diminishing the parasitoid response to the herbivore-induced odor. To test these hypotheses, we performed a series of olfactometer experiments where we measured the attraction of *C. marginiventris* females to the TPS10 or TPS8 terpene blends, either singly or in combination, as emitted from transgenic *A. thaliana* lines. We also tested whether the

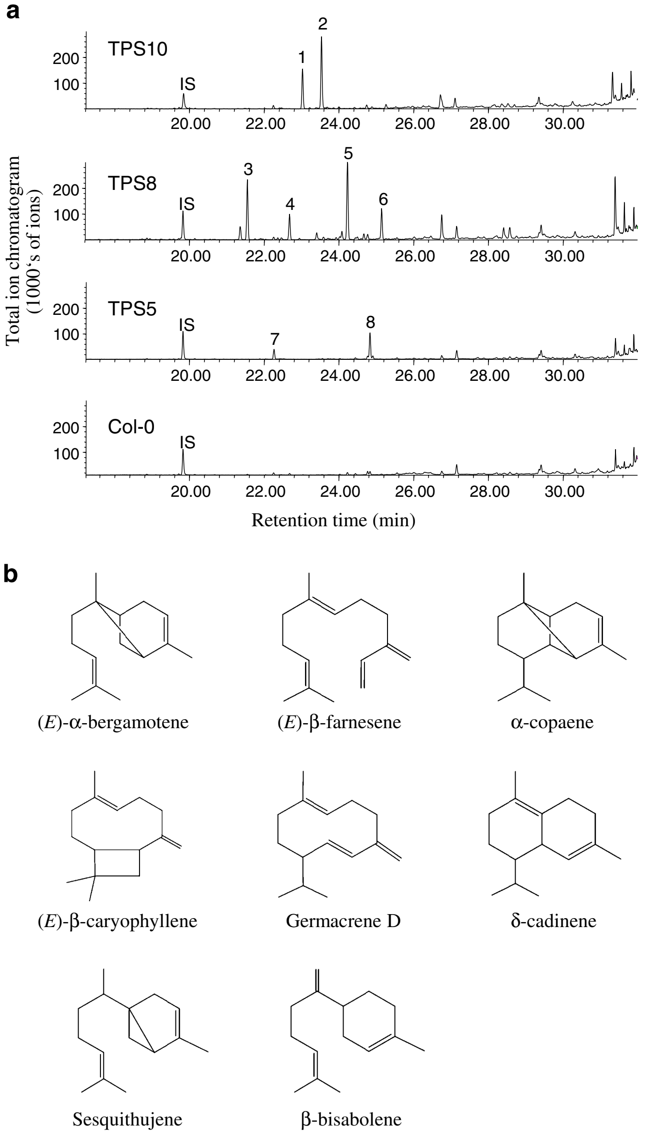

Fig. 1 **a** Volatile profile of *Arabidopsis thaliana* rosette stage plants transformed with *tps10*, *tps8*, and *tps5*, and of untransformed Col-0 plants. The main volatile compounds were identified as: 1. (*E*)-α-bergamotene; 2. (*E*)-β-farnesene; 3. α –copaene; 4. (*E*)-β-caryophyllene; 5. germacrene D; 6. δ-cadinene; 7. sesquithujene; 8. β-bisabolene. Their structures are shown in **b**. IS: internal standard

parasitoid's responses to the terpene blends are innate or learnt by association with an oviposition experience. It has been shown that *C. marginiventris* females can be attracted to different odors (among which is the odor of herbivore-induced maize) only after they had associated them to an oviposition experience (Hoballah and Turlings, 2005; Schnee et al., 2006). However, it is not known whether the parasitoids learn the whole blend or only some of its components, nor how specifically they learn the individual constituents. Finally, by using a third maize terpene blend, that of TPS5, we investigated whether the complexity of the mixture is an important variable in parasitoid attraction.

Methods and Materials

Generation of Transgenic Arabidopsis thaliana *Plants.* The generation of transgenic TPS10 (Schnee et al., 2006) and TPS8 (Fontana et al. unpublished data) plants has been described elsewhere. For TPS5 plants, the open reading frame (ORF) of *tps5* from *Zea mays* var. Delprim (*tps5-Del1*) was amplified from the pASK-IBA7 construct (Köllner et al., 2004b) with the primers forward (*tps5*fwd): 5′-ATGGCGTCTCCTCCAGCACATCG-3′ and reverse (*tps5*rvs): 5′-TCATTCGGGTATTGGCTCCACAAACAG-3′ and cloned into the pCR-TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequencing. After sequence analysis, the 1665 bp ORF was re-amplified from the sequencing vector and cloned using Gateway technology (Karimi et al., 2002) into the plant expression vector pB2GW7 between the p35S promoter and the T35S terminator of the cauliflower mosaic virus. The resulting construct was introduced into the *Agrobacterium tumefaciens* strain GV3101, which was then used to transform *Arabidopsis thaliana* L. (ecotype Col-0) plants using the floral dip method (Clough and Bent, 1998) supplemented with vacuum infiltration. Since the construct had a *bar* selectable marker gene that confers resistance to the herbicide Basta, seeds from T_0 plants were screened for the transgene by applying Basta on soil-germinated young seedlings. To select for transgenic lines emitting the expected TPS5 volatile sesquiterpenes, headspace volatiles were collected from detached leaves of the Basta survivors with SPME (solid phase microextraction) and analyzed by GC-MS (see *Volatile Collection and Analysis* for details). Transformation was confirmed additionally by PCR analysis.

Plant and Insect Material A. thaliana plants overexpressing one of the maize sesquiterpene synthases TPS10, TPS8, or TPS5 (Schnee et al., 2006; Fontana et al. unpublished data), and wild-type (ecotype Col-0) plants were grown in 7 x 7 x 8 cm single pots on fertilized potting soil in a climate chamber at 21°C, 55% relative humidity, and 150 μmol s^{-1} m^{-2} photosynthetically active radiation (PAR) under short day conditions (8 h light day^{-1}) in order to retard flowering and maximize rosette size.

Maize plants var. Delprim were grown in a climate chamber at 23±2°C, 60% r.h., 16 h light day^{-1}, and 900 μmol s^{-1} m^{-2} PAR. Eggs of the Egyptian cotton armyworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), were obtained from Syngenta rearing facilities (Stein, Switzerland). After emergence, larvae were reared on a wheatgerm-based artificial diet (also supplied by Syngenta) at room temperature. The solitary endoparasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) was reared as described in Turlings et al. (2004). Female and male adult parasitoids were kept together in plastic cages (30 x 30 x 30 cm) (MegaView Science Education Services Co. Ltd., Taiwan), at a ratio of approximately 2 : 1 in incubators at 25±1°C with 16 h light day^{-1} and supplied with moist cotton and honey. Mated, 2- to 5-d-old females were used in the olfactometer experiments.

Olfactometer Experiments The behavioral response of *C. marginiventris* females to the three maize sesquiterpene blends and their combinations was tested by using transgenic *A. thaliana* plants as odor sources in six-arm olfactometer experiments. The six-arm olfactometer (described in Turlings et al., 2004) was set with incoming air at 1.2 l min^{-1} per vessel. A portion of the air was pulled out from each vessel at 0.6 l min^{-1} through a volatile collection trap (see *Volatile Collection and Analysis*). Each of the six vessels either contained a plant as the odor source or was left empty. Purified, humidified air carried the volatile odors from the vessels to the corresponding arm of the olfactometer. When combinations of two or three terpene blends were tested, two or three *A. thaliana* plants were used as odor sources. The same number of plants was used in all the odor-carrying arms in order to have a comparable plant biomass in all the vessels. Plants used were all at the rosette stage, 4 to 6 wk-old. The transgenic plants were tested to confirm volatile emission prior to each experiment, and different confirmed individuals were chosen randomly for each experimental replicate (see *Volatile Collection and Analysis*).

Groups of 6 *C. marginiventris* females were released into the center of the olfactometer and were given 30 min to fly into one of the arms. Wasps that did not enter an arm within this time were considered as having made no choice. After the choice of the wasps had been recorded, the wasps were removed from the olfactometer. Each group of parasitoids tested was either naïve (with neither oviposition experience nor exposure to any of the tested odors) or experienced (for details on the number of releases per experience and experimental replicates, see Table 1). Oviposition experiences were given by placing a single female parasitoid in a glass enclosure that contained about 20 2nd- or 3rd-instar *S. littoralis* larvae and connected on one side to a glass vessel that contained a given odor source, which was either a transgenic *Arabidopsis* plant or a maize seedling. The wasps were allowed to oviposit in presence of the odor at least three times before they were removed from the enclosure. To induce volatile production in the maize seedling used for these experiments, plants were enclosed in a glass vial with a vented lid and one 3rd-instar *S. littoralis* caterpillar was allowed to feed overnight.

Every experiment was replicated 3 to 8 times. At the end of each replicate, all parts of the olfactometer were washed with water, acetone, and hexane, and the glass parts were dried in an oven at 250°C. In each replicate, 1 to 4 groups of 6 wasps with the same experience were released (Table 1). In total, four experiments were performed. The layout of the olfactometer for each experiment is schematically represented in Fig. 2.

Volatile Collection and Analysis All transgenic *A. thaliana* plants were screened for volatile production before being used in the olfactometer experiments. A leaf was detached from ca. 3-wk-old plants, put in a 1.5 ml glass vial, and incubated for about 20 min at 40°C with an SPME fiber (100 μm polydimethylsiloxane coating, Supelco, USA). The collected volatiles were then analyzed by GC-MS (GC: Hewlett-Packard 6890; carrier gas: He, 1 ml min^{-1}, splitless injection (injection T: 220°C), column: DB-5MS (5% diphenyl/95% dimethylsiloxane, 30 m x 0.25 mm x 0.25 μm film, J & W Scientific, Folsom, CA, USA), temperature program: 80°C to 180°C at 10°C min^{-1}, 180°C to 220°C at 100°C min^{-1}. MS: Hewlett-Packard 5973, quadrupole mass selective detector; transfer line T: 270°C; ionization potential: 70 eV; scan range: m/z 40–350).

During the second olfactometer experiment, volatiles were collected from the vessels in order to assess whether the quantity of volatiles affected the wasp

Table 1 Summary of details of the olfactometer experiments, including types of oviposition experience used for *Cotesia marginiventris* prior to experiment, number of releases per experience, number of experimental replicates, and total number of wasps used[a]

	Experiences					Releases per experience	Replicates of the experiment	Total number of wasps
	Naïve	TPS8	TPS10	TPS8 + TPS10	HI[b] maize			
Exp. 1	x	x				4	3	144
Exp. 2	x	x	x	x	x	1	8	240
Exp. 3			x			3 - 4	4	84
Exp. 4			x			5 - 6	7	216

[a] Six wasps were used in each release. Naïve wasps had no prior oviposition experience

[b] HI: Herbivore-induced

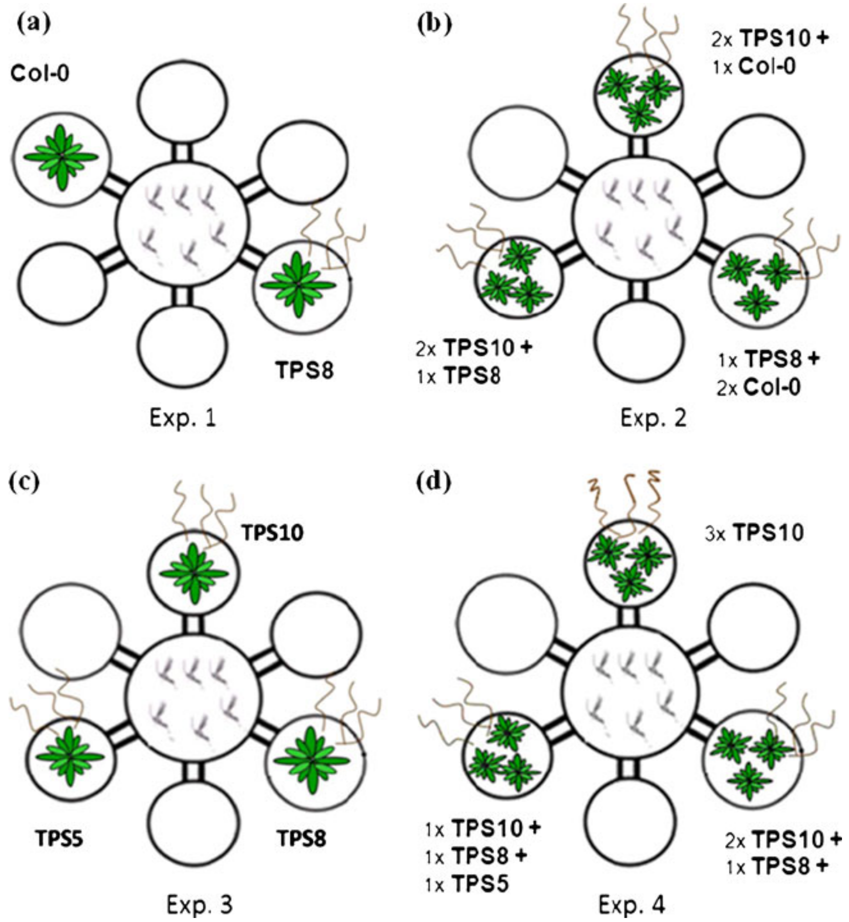

Fig. 2 Position of the odor sources in the olfactometer experiments (Experiment 1 **a**, Exp. 2 **b**, Exp. 3 **c**, Exp. 4**d**). The schematic plants represent *Arabidopsis thaliana* individuals, either transgenic or wild-type (ecotype Col-0)

responses. Odors were trapped with 25 mg Super-Q adsorbent (Alltech Associates, Inc., Deerfield, IL, USA), and eluted with 150 µl dichloromethane; 200 ng nonyl acetate were added as an internal standard. Volatiles were analyzed by GC-MS (GC: Agilent 6890 Series GC system G1530A, MS: Agilent 5973 Network Mass Selective Detector, electron impact mode, transfer line T: 230°C, ionization potential: 70 eV, scan range: m/z 33–280). The carrier gas was helium (1 ml min^{-1}). Samples were injected splitless at 230°C. We used an HP-1 column (Alltech Associates, polydimethylsiloxane, 30 m x 0.25 mm x 0.25 µm film), and the following temperature program: 40°C for 3 min, 40°C to 100°C at 8°C min^{-1}, 100°C to 200°C at 5°C min^{-1}, 5 min at 250°C. The relative quantity (%) of the sesquiterpenes was calculated based on their peak area relative to the peak area of the internal standard.

Statistical Analysis In order to analyze the influence of the odors tested and the experiences on the parasitoids' choices, a generalized linear model (GLM) with a log-link function and a quasipoisson error distribution was used (Turlings et al., 2004). "No choice" wasps were not included in the analyses. The model was fitted by maximum quasi-likelihood estimation, and its adequacy was assessed through likelihood ratio statistics and examination of residuals. Odor source and experience of the wasps were fitted to the model as fixed factors. We tested the interactions between the factors as well as the effect of the single factors on the wasps' choice. The effect of the odor was tested separately on naïve and experienced wasps in Exp. 1 and 2. The response of the wasps to the relative amounts of volatiles offered in Exp. 2 was assessed with the same GLM. All analyses were performed using the software package R (R Development Core Team (2008), R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0,URL http://www.R-project.org).

Results

The Constitutively Produced Maize Sesquiterpene Blend Is Attractive to Experienced C. marginiventris *Parasitoids (Exp.1)* Experienced *C. marginiventris* females that had had a previous oviposition experience in the presence of TPS8 volatiles were more attracted by *A. thaliana* plants producing

TPS8 terpenes than by wild-type plants ($F_{1,22}$=14.00, P=0.001), while naïve parasitoids did not show a preference for either plant ($F_{1,22}$=0.01, P=0.915) (Fig. 3a, b). Both naïve and experienced wasps preferred arms containing a plant to empty arms (naïve: $F_{2,69}$=14.40, P<0.001, experienced: $F_{2,69}$=45.63, P<0.001). Overall, 49% of the parasitoids responded to the plant odors (i.e., they entered an arm), while 45% made no choice (Fig. 3c).

Experienced C. marginiventris *Females Prefer the Full Sesquiterpene Blend of an Herbivore-Induced Maize Plant Including Constitutive Volatiles (Exp.2)* Since experienced *C. marginiventris* were attracted to the constitutively released TPS8 volatiles of a maize plant, we tested whether this attraction is similar to that of herbivore-induced TPS10 volatiles (Schnee et al., 2006) and whether there is any interaction between the two terpene blends. The volatile blend of herbivore-damaged maize seedlings contains about twice as many herbivore-induced TPS10 volatiles as constitutive TPS8 compounds. To mimic these proportions, two TPS10-emitting plants were combined with one TPS8-expressing plant (Fig. 2b, Exp.2). This blend was compared to the volatiles of two TPS10 plants combined with one Col-0 plant and to the volatiles of one TPS8 plant with two Col-0 plants to simulate the ratio of herbivore-induced to constitutive volatiles naturally emitted by maize plants. The combination of TPS10 and TPS8 sesquiterpenes was preferred by experienced wasps over the TPS8 volatiles alone (t=−2.65, P=0.009), but not over the TPS10 volatiles (t=−1.51, P=0.13) (Fig. 4b). Experienced *C. marginiventris* preferentially oriented towards arms carrying plant odors than towards empty arms ($F_{1,190}$=38.93, P<0.001). Naïve wasps did not significantly prefer any of the odors offered ($F_{2,21}$=0.85, P=0.44) (Fig. 4a), but they chose arms carrying plant volatiles more frequently than empty arms ($F_{2,21}$=0.85, P=0.03). The different oviposition experiences had no influence on the parasitoids' choice ($F_{3,188}$=0.24, P=0.87). The wasps' responsiveness was low: 40% of the parasitoids chose a plant odor, while 51% made no choice (Fig. 4c). The relative amount of the volatiles collected from the plant-containing vessels did not influence the distribution of the wasps in the olfactometer ($F_{1,22}$=6*10^{-4}, P=0.98).

Experienced C. marginiventris *Females Do Not Discriminate Between Different Maize Sesquiterpene Blends (Exp.3)* We tested the hypothesis suggested by the previous experiment that the association of volatiles with oviposition may not be dependent on specific sesquiterpenes. The parasitoids preferred arms containing a plant to empty arms ($F_{1,82}$=9.23, P=0.003), but none of the sesquiterpene blends offered was preferred over the others ($F_{2,39}$=0.58, P=0.56) (Fig. 5a). Again, the responsiveness was low: 30% of the parasitoids chose a plant odor, while 61% made no choice (Fig. 5b). These results indicated that *C. marginiventris* did not associate specific sesquiterpenes with oviposition and chose any of the three blends for host finding.

Experienced C. marginiventris *Females Tended to Orient towards More Complex Sesquiterpene Blends (Exp.4)* Because specific sesquiterpenes were not recognized by *C. marginiventris* (Exp.3), and because the blend of TPS8 and TPS10 sesquiterpenes combined were more attractive than each separately (Exp. 2), we tested whether greater complexity of the blend may increase its attraction to the wasps. Parasitoids preferred arms containing a plant to empty arms ($F_{1,95}$=6.72, P=0.011) (Fig. 6b). Although there was no statistically significant preference for any of the blends offered, the wasps tended to orient towards the most complex blend (TPS8 + TPS10 + TPS5) ($F_{2,45}$=2.67, P=0.08) (Fig. 6a). Overall, 25% of the parasitoids responded to the plant odors, while 66% made no choice.

Discussion

Herbivore-induced plant volatiles have been frequently demonstrated to play a role in the attraction of herbivore

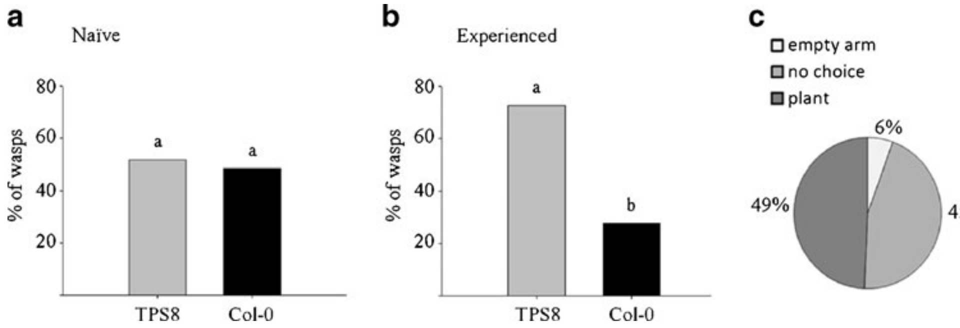

Fig. 3 Percentage of naïve **a** and experienced **b** *Cotesia marginiventris* females that were attracted to a terpene-emitting (TPS8) or a wild-type (Col-0) *Arabidopsis thaliana* plant in a six-arm olfactometer. Different letters represent a significant difference (P=0.001). Responsiveness of all the wasps tested is shown in **c**, where N=144=100%

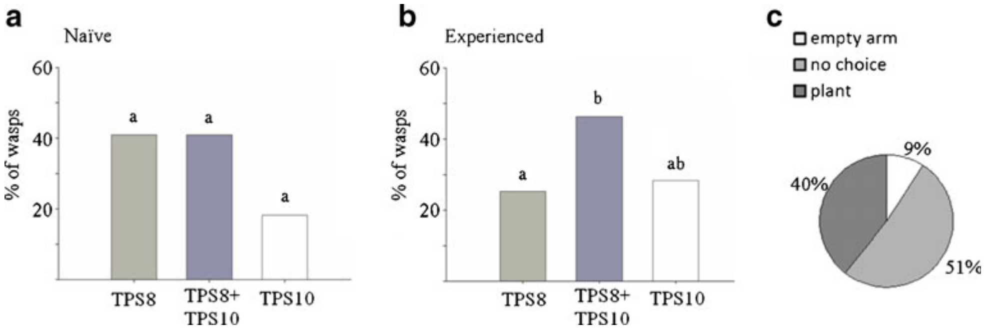

Fig. 4 Percentage of naïve **a** and experienced **b** *Cotesia marginiventris* females that were attracted to three maize sesquiterpene blends produced by transgenic *Arabidopsis thaliana* plants in six-arm olfactometer experiments. TPS8: sesquiterpene volatiles of an undamaged maize plant; TPS8 + TPS10: sesquiterpene volatiles of an herbivore-induced maize plant; TPS10: herbivore-induced sesquiterpenes of maize. Each transgenic line produces only a single blend; the mixture of TPS8 and TPS10 volatiles was obtained by combining TPS8- and TPS10-overexpressing plants. Different letters represent significant differences (P=0.009). Responsiveness of all the wasps tested is shown in **c**, where N=240=100%

enemies (Turlings et al., 1990; Takabayashi et al., 1991; Heil, 2008; Dicke, 2009). However, these studies usually do not take into account that enemies using herbivore-induced plant volatiles to search for their prey or hosts also come into contact with VOCs emitted by undamaged plant parts. A successful host finding strategy of an enemy might, therefore, involve both constitutive and herbivore-induced plant volatiles.

By utilizing *A. thaliana* plants genetically transformed with maize terpene synthases to emit blends of maize volatiles, we showed that females of *C. marginiventris*, a hymenopteran parasitoid that uses lepidopteran larvae as its hosts, can learn the constitutively released volatiles during an oviposition experience and be subsequently attracted to them. (Exp. 1, Fig. 3). Several other parasitoid species have also been shown to be attracted to odors from undamaged plants that support their hosts, including the aphid parasitoid *Aphidius funebris* Mackauer (Hymenoptera: Braconidae), which responded to uninfested *Centaurea nigra* L. (Asteraceae) (Pareja et al., 2007), and the lepidopteran parasitoids *Microplitis croceipes* and *Campoletis sonorensis*, which responded to cotton plant volatiles (Elzen et al., 1986, 1987). Here, we showed conclusively that specific maize sesquiterpene volatiles are involved in this attraction to undamaged plants.

In our experiments, experienced *C. marginiventris* females parasitoids were given a choice between terpene blends that reproduced: 1) the constitutive odor of an undamaged maize plant; 2) the odor produced by herbivore induction; or 3) the complete odor of an herbivore-infested plant, including both constitutive and induced volatiles. They showed a preference for the sesquiterpene blend that represented the combination of both constitutive and induced odors (Exp. 2, Fig. 4b). This shows that constitutively produced VOCs can act together with herbivore-induced VOCs to promote attraction of herbivore enemies. The constitutively emitted TPS8 terpenes may represent a background odor that indicates the general habitat of the host larvae. This background odor appears to reinforce the particular odor released by maize only upon herbivore feeding, thus enhancing parasitoid attraction. Such a positive

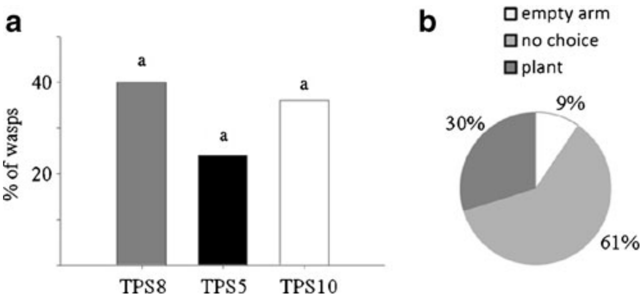

Fig. 5 Percentage of *Cotesia marginiventris* females that were attracted to sesquiterpene blends from maize produced by three lines of transgenic *Arabidopsis thaliana* plants in six-arm olfactometer experiments **a**. The terpene composition of the blends is detailed in Fig. 1. Responsiveness of all the wasps tested is shown in **b**, where N=240=100%

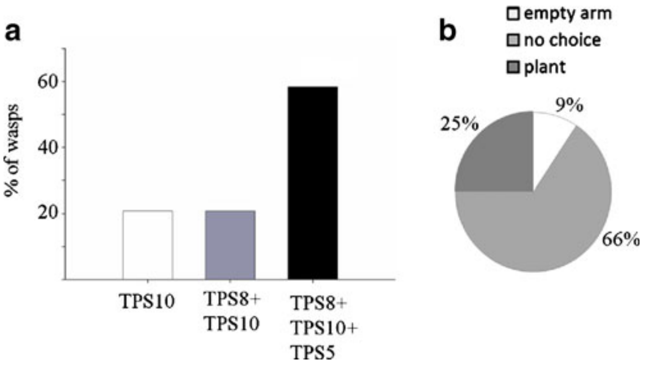

Fig. 6 Percentage of *Cotesia marginiventris* females that were attracted to sesquiterpene blends of increasing complexity in six-arm olfactometer experiments **a**. The odor sources were *Arabidopsis thaliana* plants each transformed with one maize sesquiterpene synthase gene (*tps8* or *tps10* or *tps5*). Plants from different transgenic lines were combined to give the combinations of odor blends. Responsiveness of all the wasps tested is shown in **b**, where N=96=100%

interaction of volatiles cues also has been described for *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillault) (Hymenoptera: Eucoilidae), a larval parasitoid of *Drosophila* species, which is attracted to the odors of pear and banana fruits (Couty et al., 1999). When this odor was provided together with damp filter papers impregnated with the smell of *Drosophila* flies, the parasitoids significantly preferred this combination to the smell of the flies alone. The importance of background odor for parasitoid host searching also has been highlighted by Mumm and Hilker (2005) in their study with the egg parasitoid *Closterocerus ruforum*, which did not respond to the signal of oviposition-induced pine twigs ((*E*)-β-farnesene) unless it was offered in combination with the odor of an uninfested twig. Another interesting interaction between odor blends has been reported by Fukushima and coworkers (2002), who found a synergistic effect in the attraction of conditioned *Cotesia kariyai* (Hymenoptera: Braconidae) parasitoids to a blend of four volatiles typically emitted after herbivory (geranyl acetate, (*E*)-β-caryophyllene, (*E*)-β-farnesene, and indole), and a non-specific blend made up of three GLVs, β-myrcene, and linalool. A limitation of this and other studies on volatiles lies in the authenticity of such partial plant volatile blends that are artificially constructed by mixing purified compounds, and may lack critical minor compounds. The use of transgenic *A. thaliana* plants transformed with TPS genes resulted in the formation of both major and minor components of the sesquiterpene blend, allowing a more accurate evaluation of its ecological function. Minor constitutents of volatile blends sometimes have been shown to play a disproportionate role in insect attraction (Leal, 2005, and references therein). In the present study, minor constituents other than sesquiterpenes could conceivably influence parasitoid attraction, but these did not differ among the *A. thaliana* lines tested (Suppl. Fig. 1).

The preference of experienced *C. marginiventris* females for the combination of two terpene blends observed in Experiment 2 also could be explained by the fact that more complex mixtures of terpenes are more attractive to these wasps. We tested, therefore, the effect of mixture complexity on *C. marginiventris* by giving females the choice between three terpene blends with an increasing number of components (Exp. 4). Here, the parasitoids tended to orient towards the most complex blend, suggesting that the number of compounds of a terpene mixture may play a role in attraction. Similar results have been obtained in field experiments where the number of insect herbivores attracted by a volatile blend was positively correlated with the number of chemicals present in the blend (Szendrei and Rodriguez-Saona, 2010). Complex volatile blends are known to play important roles in plant-insect interactions besides attraction of herbivore enemies. In a recent study, Riffell and coworkers investigated the perception and behavioral response of the moth *Manduca sexta* L. (Lepidoptera: Sphingidae) to the floral volatiles of the Sacred Datura (*Datura wrightii* (Regel), Solanaceae) (Riffell et al., 2009). Of the over 60 components of the floral scent, many of them terpenes, nine elicited a neural response in the moth, and, among these, four were monoterpenes (linalool, nerol, β-myrcene, and geraniol), and two were sesquiterpene hydrocarbons ((*E*)-β-caryophyllene and (*E,E*)-α-farnesene). Strikingly, these nine compounds were attractive to the moths only when offered as a mixture, but not when tested individually.

Theoretically, the preference of experienced *C. marginiventris* for the combination of two sesquiterpene blends observed in Experiment 2 also could be due to a higher total amount of volatiles, which leads to a stronger response. Previous studies indicate that the quantity of odors can be positively correlated to the attractiveness for different parasitoid species (Vaughn et al., 1996; Hoballah et al., 2002; Turlings et al., 2004; Kugimiya et al., 2010). However, in our work, the abundance of volatiles released in the olfactometer did not appear to influence the choice of the parasitoid (see *Results*, Exp. 2).

The parasitoid wasps used in our experiments did not prefer the TPS10 sesquiterpene mixture that they were trained on over the sesquiterpene mixtures formed by TPS8 and TPS5, although there are no major sesquiterpenes common to the three odors. This suggests that *C. marginiventris* may be attracted by the presence of sesquiterpenes in general, and does not distinguish among individual compounds. A study on *M. croceipes* investigated the ability of this egg parasitoid to distinguish among aliphatic alcohols differing in carbon chain-length and the position of functional groups (Meiners et al., 2002). The results suggested that a difference of at least two C atoms is necessary for wasps to make a choice between two aliphatic alcohols. It is possible that *C. marginiventris*, like *M. croceipes*, does not distinguish among mixtures of sesquiterpene hydrocarbons that all contain 15 carbon atoms, and, therefore, did not choose among the different blends offered, once they had learnt one of them.

The responsiveness of the parasitoids to the odors offered seemed to be low in our experiments, ranging from 25-49% (Figs. 3b, 4c, 5b, 6b). However, a responsiveness of *C. marginiventris* females of 42% was recorded previously by Turlings et al. (2004) in experiments with a six-arm olfactometer. An even lower responsiveness was found by Girling et al. (2006) in Y-tube olfactometer experiments with an aphid parasitoid (*Diaretiella rapae* (M'Intosh) (Hymenoptera: Aphiididae)) and *A. thaliana* plants. In this study, the percentages of parasitoids responding to the odors offered were as low as 22.5%. From this result, the authors concluded that the corresponding odors were not especially attractive to *D.*

rapae. On the other hand, Schnee et al. (2006) reported a responsiveness of 83% in olfactometer experiments with *C. marginiventris* and sesquiterpene-emitting TPS10 *A. thaliana* plants. Such a high responsiveness compared to the one we recorded could be due to the fact that the experiments were performed at different times of the year (Schnee et al. (2006) in spring, the experiments presented here in summer or in winter).

Studies on naïve *C. marginiventris* females that utilize the complete herbivore-induced volatiles of a maize plant demonstrated a responsiveness of up to 80% (D'Alessandro and Turlings, 2005). These high rates of attraction to the complete blend might be due to the fact that this contained one or more volatile compounds not present in our blends that may not have even been identified yet (D'Alessandro and Turlings, 2005; D'Alessandro et al., 2009). To fully understand the importance of volatile compounds in the host finding of *C. marginiventris*, bioassays should be carried out with additional constitutive host volatiles, such as monoterpenes and GLVs. Further tests with other parasitoids with different degrees of host specialization should give even more insight into the relative importance of constitutive vs. herbivore-induced compounds.

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References

BRILLI, F., CICCIOLI, P., FRATTONI, M., PRESTININZI, M., SPANEDDA, A. F., and LORETO, F. 2009. Constitutive and herbivore-induced monoterpenes emitted by *Populus* x *euroamericana* leaves are key volatiles that orient *Chrysomela populi* beetles. *Plant Cell Environ.* 32:542–552.

CLOUGH, S. J. and BENT, A. F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735–743.

COUTY, A., KAISER, L., HUET, D., and PHAM-DELEGUE, M. H. 1999. The attractiveness of different odour sources from the fruit-host complex on *Leptopilina boulardi*, a larval parasitoid of frugivorous *Drosophila* spp. *Physiol. Entomol.* 24:76–82.

D'ALESSANDRO, M., BRUNNER, V., VON MEREY, G., and TURLINGS, T. C. J. 2009. Strong attraction of the parasitoid *Cotesia marginiventris* towards minor volatile compounds of maize. *J. Chem. Ecol.* 35:999–1008.

D'ALESSANDRO, M. and TURLINGS, T. C. J. 2005. *In situ* modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chem. Senses* 30:739–753.

DE BOER, J. G. and DICKE, M. 2004. Experience with methyl salicylate affects behavioural responses of a predatory mite to blends of herbivore-induced plant volatiles. *Entomol. Exp. Appl.* 110:181–189.

DE BOER, J. G., POSTHUMUS, M. A., and DICKE, M. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or nonprey herbivores by a predatory mite. *J. Chem. Ecol.* 30(11):2215–2230.

DICKE, M. 2009. Behavioural and community ecology of plants that cry for help. *Plant Cell Environ.* 32:654–665.

DICKE, M., VAN LOON, J. J. A., and SOLER, R. 2009. Chemical complexity of volatiles from plants induced by multiple attack. *Nature Chem. Biol.* 5:317–324.

DUDAREVA, N., PICHERSKY, E., and GERSHENZON, J. 2004. Biochemistry of plant volatiles. *Plant Physiol.* 135:1893–1902.

ELZEN, G. W., WILLIAMS, H. J., and VINSON, S. B. 1986. Wind tunnel flight responses by hymenopterous parasitoid *Campoletis sonorensis* to cotton cultivars and lines. *Entomol. Exp. Appl.* 42:285–289.

ELZEN, G. W., WILLIAMS, H. J., VINSON, S. B., and POWELL, J. E. 1987. Comparative flight behavior of parasitoids *Campoletis sonorensis* and *Microplitis croceipes*. *Entomol. Exp. Appl.* 45:175–180.

FUKUSHIMA, J., KAINOH, Y., HONDA, H., and TAKABAYASHI, J. 2002. Learning of herbivore-induced and nonspecific plant volatiles by a parasitoid, *Cotesia kariyai*. *J. Chem. Ecol.* 28:579–586.

GIRLING, R. D., HASSALL, M., TURNER, J. G., and POPPY, G. M. 2006. Behavioural responses of the aphid parasitoid *Diaeretiella rapae* to volatiles from *Arabidopsis thaliana* induced by *Myzus persicae*. *Entomol. Exp. Appl.* 120:1–9.

HATANO, E., KUNERT, G., MICHAUD, J. P., and WEISSER, W. W. 2008. Chemical cues mediating aphid location by natural enemies. *Eur. J. Entomol.* 105:797–806.

HEIL, M. 2008. Indirect defence via tritrophic interactions. *New Phytol.* 178:41–61.

HOBALLAH, M. E. and TURLINGS, T. C. J. 2005. The role of fresh versus old leaf damage in the attraction of parasitic wasps to herbivore-induced maize volatiles. *J. Chem. Ecol.* 31:2003–2018.

HOBALLAH, M. E. F., TAMO, C., and TURLINGS,T. C. J. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: Is quality or quantity important? *J. Chem. Ecol.* 28:951–968.

KARIMI, M., INZE, D., and DEPICKER, A. 2002. GATEWAY((TM)) vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* 7:193–195.

KESSELMEIER, J. and STAUDT, M. 1999. Biogenic volatile organic compounds (VOC): An overview on emission, physiology and ecology. *J. Atmos. Chem.* 33:23–88.

KÖLLNER, T. G., SCHNEE, C., GERSHENZON, J., and DEGENHARDT, J. 2004a. The sesquiterpene hydrocarbons of maize (*Zea mays*) form five groups with distinct developmental and organ-specific distribution. *Phytochemistry* 65:1895–1902.

KÖLLNER, T. G., SCHNEE, C., GERSHENZON, J., and DEGENHARDT, J. 2004b. The variability of sesquiterpenes cultivars emitted from two *Zea mays* cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes. *Plant Cell* 16:1115–1131.

KÖLLNER, T. G., SCHNEE, C., LI, S., SVATOS, A., SCHNEIDER, B., GERSHENZON, J., and DEGENHARDT, J. 2008. Protonation of a neutral (*S*)-beta-bisabolene intermediate is involved in (*S*)-beta-macrocarpene formation by the maize sesquiterpene synthases TPS6 and TPS11. *J. Biol. Chem.* 283:20779–20788.

KÖLLNER, T. G., GERSHENZON, J., and DEGENHARDT, J. 2009. Molecular and biochemical evolution of maize terpene synthase 10, an enzyme of indirect defense. *Phytochemistry* 70:1139–1145.

KUGIMIYA, S., SHIMODA, T., TABATA, J., and TAKABAYASHI, J. 2010. Present or past herbivory: a screening of volatiles released from *Brassica rapa* under caterpillar attacks as attractants for the solitary parasitoid, *Cotesia vestalis*. *J. Chem. Ecol.* 36:620–628.

LEAL, W. S. 2005. Pheromone reception, pp. 1–36, in S. Schulz (ed.). Chemistry of Pheromones and other Semiochemicals II. Springer-Verlag, Berlin.

MEINERS, T., WACKERS, F., and LEWIS, W. J. 2002. The effect of molecular structure on olfactory discrimination by the parasitoid *Microplitis croceipes*. *Chem. Senses* 27:811–816.

MUMM, R. and HILKER, M. 2005. The significance of background odour for an egg parasitoid to detect plants with host eggs. *Chem. Senses* 30:337–343.

PAREJA, M., MORAES, M. C. B., CLARK, S. J., BIRKETT, M. A., and POWELL, W. 2007. Response of the aphid parasitoid *Aphidius funebris* to volatiles from undamaged and aphid-infested *Centaurea nigra*. *J. Chem. Ecol.* 33:695–710.

RIFFELL, J. A., LEI, H., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 2009. Characterization and coding of behaviorally significant odor mixtures. *Curr. Biol.* 19:335–340.

RUTLEDGE, C. E. 1996. A survey of identified kairomones and synomones used by insect parasitoids to locate and accept their hosts. *Chemoecology* 7121–131.

SCHNEE, C., KÖLLNER, T. G., HELD, M., TURLINGS, T. C. J., GERSHENZON, J., and DEGENHARDT, J. 2006. The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. *Proc. Natl. Acad. Sci. USA* 103:1129–1134.

SZENDREI, Z. and RODRIGUEZ-SAONA, C. 2010. A meta-analysis of insect pest behavioral manipulation with plant volatiles. *Entomol. Exp. Appl.* 134:201–210.

TAKABAYASHI, J., NODA, T., and TAKAHASHI, S. 1991. Plants produce attractants for *Apanteles kariyai*, a parasitoid of *Pseudaletia separata* - Cases of communication and misunderstanding in parasitoid-plant interactions. *Appl. Entomol. Zool.* 26:237–243.

TURLINGS, T. C. J. and TON, J. 2006. Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. *Curr. Opin. Plant Biol.* 9:421–427.

TURLINGS, T. C. J., DAVISON, A. C., and TAMO, C. 2004. A six-arm olfactometer permitting simultaneous observation of insect attraction and odour trapping. *Physiol. Entomol.* 29:45–55.

TURLINGS, T. C. J., TUMLINSON, J. H., HEATH, R. R., PROVEAUX, A. T., and DOOLITTLE, R. E. 1991. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17:2235–2251.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

UNSICKER, S. B., KUNERT, G., and GERSHENZON, J. 2009. Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. *Curr. Opin. Plant Biol.* 12:479–485.

VAUGHN, T. T., ANTOLIN, M. F., and BJOSTAD, L. B. 1996. Behavioral and physiological responses of *Diaeretiella rapae* to semiochemicals. *Entomol. Exp. Appl.* 78:187–196.