LETTER

Maize landraces recruit egg and larval parasitoids in response to egg deposition by a herbivore

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Abstract

Natural enemies respond to herbivore-induced plant volatiles (HIPVs), but an often overlooked aspect is that there may be genotypic variation in these 'indirect' plant defence traits within plant species. We found that egg deposition by stemborer moths (*Chilo partellus*) on maize landrace varieties caused emission of HIPVs that attract parasitic wasps. Notably, however, the oviposition-induced release of parasitoid attractants was completely absent in commercial hybrid maize varieties. In the landraces, not only were egg parasitoids (*Trichogramma bournieri*) attracted but also larval parasitoids (*Cotesia sesamiae*). This implies a sophisticated defence strategy whereby parasitoids are recruited in anticipation of egg hatching. The effect was systemic and caused by an elicitor, which could be extracted from egg materials associated with attachment to leaves. Our findings suggest that indirect plant defence traits may have become lost during crop breeding and could be valuable in new resistance breeding for sustainable agriculture.

Keywords

Induced defense, insect oviposition, multitrophic interaction, plant volatiles, *Zea mays*.

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INTRODUCTION

Under natural conditions, plants have evolved direct and indirect defence strategies against attacking organisms (Sabelis *et al.* 1999; Howe & Jander 2008). Directly, they produce toxins, digestion inhibitors and herbivore-induced plant volatiles (HIPVs) repellent to phytophagous insects (Duffey & Stout 1996; De Moraes *et al.* 2001; Kessler & Baldwin 2001); indirectly, they use HIPVs to attract natural enemies antagonistic to the herbivores (Turlings *et al.* 1990; Loughrin *et al.* 1995; De Moraes *et al.*1998; Dicke & van Loon 2000; Heil 2008). However, these naturally occurring defence responses may have been lost whilst selective breeding favoured other traits such as yield (Sotelo 1997; Migui & Lamb 2003; Köllner *et al.* 2008). Landraces comprise locally adapted crop germplasm maintained by farmers rather than by breeders. The current study tests the hypothesis that emission of HIPVs following insect egg deposition may be more prevalent in landraces than in mainstream maize (corn) (*Zea mays* L.) cultivars. In maize, HIPV-mediated indirect defences are known to play an important role following larval feeding (Tumlinson *et al.* 1993; Turlings *et al.* 1998; Ngi-Song *et al.* 2000; Köllner *et al.* 2004), but there are no previous reports of maize responses to egg deposition by herbivores and associated tritrophic interactions.

The spotted stemborer, *Chilo partellus* Swinhoe (Lepidoptera: Crambidae), is a major insect pest of maize in eastern and southern Africa and South Asia, causing yield losses of up to 88% (Kfir *et al.* 2002). Since its introduction in Africa early in the last century (Tams 1932), it has spread to many different agroecological zones (Kfir *et al.* 2002; Tamiru *et al.* 2007) and has proved to be a very efficient coloniser and a devastating pest wherever it occurs (Kfir 1997; Tamiru

et al. 2011). Use of insecticides for pest control is not only expensive, in the context of smallholder farmers, but may also have undesirable consequences such as resistance development, secondary pest outbreaks, environmental pollution and risk to spray operators (Bruce 2010). Furthermore, stemborer larvae are difficult spray targets as they are hidden within the plant stem. Whilst Bt maize has considerable potential (Estruch *et al.* 1997), it is not yet available to the majority of African smallholders, who have limited access even to the improved varieties developed in the Green Revolution (Borlaug 2007). The ecology of herbivore/plant interactions is therefore being studied to develop cost-effective and environmentally benign alternative control options that make use of natural plant defence responses, which would be highly relevant for resource-poor African farmers.

For agriculture, no plant family is as important as the Poaceae (Gramineae). Cereals provide our basic diet and grasses are the main food for our livestock. Africa is the centre of genetic diversity for one of the most important cereals, sorghum [*Sorghum bicolor* (L.) Moench], and possesses a high degree of biodiversity for many other important cereals and tropical pasture or fodder grasses. Many wild relatives and landraces of grass species from which crop plants and fodder crops have been selected continue to survive today. These may possess defence traits absent in mainstream crop cultivars. Certain African poaceous plants have sophisticated responses to herbivory that involve multitrophic interactions with natural enemies (e.g. Bruce *et al.* 2010). Furthermore, a valuable trait was discovered in the African molasses grass (*Melinis minutiflora* Beauv.), which defends itself against pest attack by constitutively releasing volatile semiochemicals that have dual effects: (i) repelling the pests and (ii) attracting the pests' natural enemies (Khan *et al.* 1997). These properties have been used in

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developing a 'push-pull' cropping system for resource-poor farmers in Africa who do not use pesticides (Cook *et al.* 2007; Khan *et al.* 2010).

Here, we examined responses of open-pollinated landrace and commercial hybrid varieties of maize to egg deposition by *C. partellus*. Landraces maintain additional diverse traits that may be of great importance for crop improvement. Any defences elicited by the presence of eggs would indicate a finely tuned and coevolved defence response sensitive even to the earliest stage of herbivore attack (Hilker & Meiners 2006; Bruce *et al.* 2010). We collected volatiles from different maize varieties, exposed to stemborer oviposition or unexposed, identified differences in the profiles of volatiles emitted, and investigated electrophysiological and behavioural responses of two key natural enemies, the egg parasitoid *Trichogramma bournieri* Pintureau (Hymenoptera: Trichogrammatidae) and the larval parasitoid *Cotesia sesamiae* Cameron (Hymenoptera: Braconidae). Our results revealed that egg deposition on maize caused emission of HIPVs attracting both egg and larval parasitoids. This effect was also shown to act systemically. From the maize varieties tested, three landraces showed increased production of these compounds, with no such effect being noted for two commercial hybrid varieties. We are not aware of any previous studies showing that oviposition-induced volatile emissions attract larval parasitoids and this is also the first time that this phenomenon has been demonstrated for an economically important crop.

MATERIALS AND METHODS

Plants

Landrace varieties of maize, *Z. mays*, obtained from the International Maize and Wheat Improvement Center (CIMMYT), and hybrid varieties from commercial seed suppliers (Western Seed Company Ltd., Kenya Seed Company Ltd.), were grown individually in pots filled with fertilised soil in an insect-proof screen house at *icipe*-Thomas Odhiambo campus, Mbita Point (0°25′S, 34°12′E; *c.* 1200 m above sea level), western Kenya. All plants were grown under natural conditions (*c.* 25 °C, 65% RH; 12L: 12D) and used in the experiments when 3–4 weeks old.

Insects

Field-collected stemborers, *C. partellus*, were reared on a semi-synthetic diet containing sorghum, *S. bicolor* (Ochieng *et al.* 1985). The egg parasitoid *T. bournieri* and the larval parasitoid *C. sesamiae* were reared on stemborer eggs and larvae respectively, using methodologies described previously (Overholt *et al.* 1994). Experimental insects were maintained at the insect mass rearing unit of *icipe*-Thomas Odhiambo campus (24 ± 3 °C, 70 ± 5% RH, 12L: 12D). The mass-reared culture was infused with a field-collected insect population every 3 months to avoid genetic decay and maintain the original behavioural characteristics of the species. Näive mated female parasitoids obtained from the fourth to fifth generation were used in the experiments.

Volatile collection

Volatile compounds from whole maize plants, with and without stemborer eggs, were collected by headspace sampling (Agelopoulos *et al.* 1999) for use in subsequent bioassays and electrophysiological and chemical analyses. Prior to volatile collection, seedlings (3–4 weeks old)

were placed inside oviposition cages (80 × 40 × 40 cm) into which six gravid female stemborer moths were introduced and kept overnight for oviposition. Concurrently, control plants were kept inside similar cages, but without stemborer moths. Volatiles were collected the following day, starting at the last 2 h of photophase, for 48 h. Leaves of plants with or without eggs were enclosed in polyethyleneterephthalate (PET) bags (volume 3.2 L, ~12.5 mm thickness) heated to 150 °C before use and fitted with Swagelock inlet and outlet ports. Charcoal-filtered air was pumped (600 mL min⁻¹) through the inlet port. Volatiles were collected on Porapak Q (0.05 g, 60/80 mesh; Supelco) filters inserted in the outlet port through which air was drawn at 400 mL min⁻¹. After entrainment, volatiles were eluted with 0.5 mL dichloromethane. To investigate systemic production of HIPVs, *C. partellus* was allowed to oviposit only on the lower leaves of maize seedlings, the upper five to seven leaves being covered with a PET bag to prevent egg deposition and the exchange/adsorption of volatiles released from the lower leaves. Volatiles from the upper (enclosed) leaves were then collected as described above. Control plants were kept under similar conditions, but without exposure to *C. partellus*.

Bioassays

A four-arm olfactometer (Pettersson 1970) was used to investigate parasitoid responses to headspace samples of volatiles and a Y-tube olfactometer (Steinberg *et al.* 1992) was used to investigate responses to odours drawn directly from whole plants.

Four-arm olfactometer bioassay

Responses of parasitoids to volatiles were tested in a Perspex four-arm olfactometer (Pettersson 1970). Air was drawn through the four arms towards the centre at 260 mL min⁻¹. Headspace samples (10 μL aliquots) were applied, using a micropipette (Drummond 'microcap', Drummond Scientific Co., Broomall, PA, USA), to a piece of filter paper (4 × 25 mm) subsequently placed in an inlet port at the end of each olfactometer arm. Mated female parasitoids, without previous exposure to plants or hosts, were transferred individually into the central chamber of the olfactometer using a custom-made piece of glass tubing. Time spent in each olfactometer arm was recorded with 'Olfa' software (F. Nazzi, Udine, Italy) for 12 min. The experiments were replicated at least 12 times. A choice test was carried out to compare insect responses to headspace samples from oviposition-induced and control plants. The two opposite arms held the test stimuli (10 μL aliquots of headspace sample). This dose was approximately equal to that emitted by 12 plants over 10 min). The remaining two arms were solvent controls. Authentic standards of synthetic compounds (1 μg dose) were also tested. The synthetic compound was applied in one of the arms, whilst the remaining three arms were blank controls (solvent only). Finally, we tested a blend of synthetic HIPVs formulated using the same ratio and concentration as found in an attractive sample.

Parasitoid responses to volatiles from maize lines treated with egg material

To investigate the effect of egg material in inducing plant defence responses, female *C. partellus* were allowed to lay eggs on microscope slides overnight. Each slide typically had 10–20 egg batches with up to 30 eggs per batch. The adhesive material attaching the eggs to the slides was extracted in absolute ethanol (1 mL, for 30 min) after

peeling the eggs off the slide with a scalpel blade, taking care not to damage the eggs. Plants were treated with a standard dose of 100 µL extract. A 10 µL drop of extract was applied to each of 10 different plant leaves, to mimic natural egg laying sites of *C. partellus*, using a micropipette (Drummond 'microcap'). Control plants were kept under the same conditions, but treated with the extraction solvent (ethanol) alone. The application was made between 4:30 and 5:30pm and volatiles were collected, using standard methods described above (i.e. Agelopoulos *et al.* 1999), from the plants the following day, for 48 h, starting at the last 2 h of photophase. The volatiles were then eluted from the Porapak Q filters with 0.5 mL dichloromethane and kept at −20 °C until used in bioassays and chemical analyses. Locally and systemically collected headspace samples from selected maize lines were tested for their attractiveness to female *C. sesamiae* in the four-arm olfactometer. During systemic application, the ethanolic egg material extract was applied only on the lower leaves of maize seedlings, while the upper five to seven leaves were covered with a PET bag to avoid possible exchange/adsorption of volatiles released from the site of application (lower leaves).

Y-tube olfactometer bioassay

The Y-tube olfactometer was as described previously (Steinberg *et al.* 1992). Live 6-week-old maize seedlings were contained in Perspex chambers (30 × 30 × 120 cm), which had the open end submerged in 15 cm water held in a plastic basin to make an airtight system. Charcoal-filtered air was pushed through air inlets (1.5 L min^{-1} each) at the bottom of the two chambers and simultaneously drawn into the Y-tube fields in the closed system by a vacuum pump (Cole-Parmer Air-Cadet, Vernon Hills, IL, USA). Larval parasitoids (*C. sesamiae*) were released individually at the stem of the Y-tube and allowed 5 min to make choices. A choice was recorded when a parasitoid remained for more than 15 s across the finishing line (4 cm past the intersection). All tests were replicated three times with 20 parasitoids per replicate.

Gas chromatographic (GC) analysis

GC analysis was carried out by injecting 2 µL of headspace sample onto a non-polar (HP-1, 50 m, 0.32 mm i.d., 0.52 µm) capillary column using an Agilent 6890 GC equipped with a cold on-column injector and flame ionisation detector (FID). The oven was maintained at 30 °C for 2 min and then programmed at 5 °C min^{-1} to 250 °C. Quantification was carried out by calculating and comparing peak areas with known amounts of authentic external standards. The stereochemistry of linalool was determined using an HP5890 GC equipped with a cool on-column injector and a FID, fitted with a β-cyclodextrin chiral capillary column (Supelco, 30 m × 0.25 mm i.d., 0.25 µm film thickness). The GC oven was maintained at 40 °C for 1 min and then raised by 5 °C min^{-1} to 150 °C, where it was held for 30 min. After confirming that successful separation of synthetic enantiomers was accomplished, co-injections were carried out. Peak enhancement confirmed the presence of the enantiomer in the headspace sample.

Electrophysiological analysis

Coupled GC-electroantennography (GC-EAG) was carried out using antennae of female *C. sesamiae* and attractive headspace samples of maize. EAG recordings were made using Ag–AgCl glass electrodes filled with saline solution [composition as in (Maddrell 1969), but without glucose]. A female parasitoid was chilled for 1 min and the head excised, and the tips of both antennae were removed to ensure a good contact. The indifferent electrode was placed within the head capsule. Signals were passed through a high impedance amplifier (UN-06; Syntech, Hilversum, The Netherlands) and analysed using a customised software package (Syntech). The GC-EAG system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams 1990). Separation of the volatiles was achieved on a GC (Agilent Technologies, 6890N) equipped with a cold on-column injector and a FID using a HP-1 column (50 m, 0.32 mm ID, 0.52 µm film thickness). The oven temperature was maintained at 30 °C for 2 min and then programmed at 15 °C min^{-1} to 250 °C. The carrier gas was helium. Outputs from the EAG amplifier and the FID were monitored simultaneously and analysed using the Syntech software package. Peaks eluting from the GC column were judged to be active if they elicited EAG activity in three or more of five coupled runs.

Authentic standards were tested using a delivery system which employed a filter paper strip in a disposable Pasteur pipette cartridge (Wadhams 1982). The stimulus (2 s duration) was delivered into a purified airstream (1 L min^{-1}) flowing continuously over the preparation using a stimulus controller (Syntech CS02). Samples (10 µL) of the standard solutions of test compounds (1 mg mL^{-1} in redistilled hexane) were applied in the cartridge. The control stimulus was hexane (10 µL). Five replicates were done. Electrophysiological responses were recorded using Syntech software.

GC-MS analysis

Aliquots of attractive headspace samples were analysed on a capillary GC column (HP-1, 50 m, 0.32 mm i.d., 0.52 µm) directly coupled to a mass spectrometer (VG Autospec; Fisons Instruments, Manchester, UK) equipped with a cool on-column injector. Ionisation was performed by electron impact (70 eV, 250 °C). The oven temperature was maintained at 30 °C for 5 min and then programmed at 5 °C min^{-1} to 250 °C. Tentative GC-MS identifications were confirmed by peak enhancement with authentic standards on two GC columns of different polarities (non-polar, HP-1 column, 50 m, 0.32 mm i.d., 0.52 µm film thickness; polar DB-wax column, 30 m, 0.32 mm i.d., 0.5 µm film thickness).

Chemicals

(*R*)-Linalool, methyl salicylate, decanal, methyleugenol, (*E*)-(1*R*,9*S*)-caryophyllene, (*Z*)-3-hexenyl acetate, α-copaene and pentadecane were purchased from Sigma Aldrich and Avocado Research Chemicals. (*E*)-Ocimene was synthesised in high purity (> 95% by GC) in two steps *via* functionalisation of 3-methyl-3-sulpholene with 1-bromo-3-methylbut-2-ene (Chou *et al.* 1984) and extrusion of sulphur dioxide using lithium aluminium hydride (Gaoni 1977). (*E*)-ß-Farnesene (> 98%) was synthesised in one step from (*E*)-farnesyl chloride (Kang *et al.* 1987). (*E*)-4,8-Dimethyl-1,3,7-nonatriene (> 98%) and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (> 98%) were synthesised from geraniol and (*E*,*E*)-farnesol respectively, by oxidation to their corresponding aldehydes followed by Wittig methylenation (Leopold 1990).

Statistical analysis

Bioassay data from the four-arm olfactometer, i.e. time spent in each arm, were compared by analysis of variance (ANOVA) after conversion of the data into proportions and a logratio transformation. Means were separated using Fisher's LSD test with α set at 0.05 (Genstat version 10, VSN International, Hemel Hempstead, UK).The Y-tube bioassay data were analysed by a chi-square goodness-of-fit test. The EAG responses of *C. sesamiae* to authentic standards were compared with the control (hexane) by fitting the data in a linear mixed model with authentic standards as the fixed component and *C. sesamiae* (rep)/authentic standard as the random component. Response to control and test solutions were compared using students' *t*-test.

RESULTS

Behavioural responses of parasitoids to headspace samples of volatiles from maize with and without eggs

Females of the egg parasitoid *T. bournieri* were significantly attracted to HIPVs from maize landrace C-2101 (Cuba) ($F_{2,34} = 7.24$, $P = 0.002$) exposed to *C. partellus* eggs, compared with volatiles from unexposed plants and blank controls (Fig. 1a). Furthermore, the oviposition-induced volatiles from landrace lines C-2101, B-3016 (Brazil) and H-2034 (Haiti) were attractive to the larval parasitoid *C. sesamiae* (C-2101: $F_{2,34} = 31.52$, $P < 0.001$; B-3016: $F_{2,34} = 18.79$, $P < 0.001$; H-2034: $F_{2,34} = 8.56$, $P < 0.001$) (Fig. 1b). In contrast, volatiles collected from two standard commercial hybrid varieties [Western Seed Company Ltd (WS505) and Kenya Seed Company Ltd (PH4)] after egg deposition were not attractive to *T. bournieri* (WS505: $F_{2,34} = 2.17$, $P = 0.13$; PH4: $F_{2,34} = 0.03$; $P = 0.967$) (Fig. 1a) or *C. sesamiae* (WS505: $F_{2,34} = 3.13$, $P = 0.056$; PH4: $F_{2,34} = 0.72$, $P = 0.495$) (Fig. 1c). Responses of adult *C. sesamiae* to plant volatile cues emitted even before their prey hatched were particularly interesting and we decided to focus our study on this species.

In addition to bioassays with headspace samples, behavioural responses to live plants bearing *C. partellus* eggs were observed in a Y-tube olfactometer bioassay. *C. sesamiae* was significantly attracted to HIPVs from oviposition-exposed landrace plants (C-2101:

$\chi^2 = 10.667$, $P = 0.0011$; H-2034: $\chi^2 = 8.3208$, $P = 0.0039$) (Fig. S1a) when offered in a choice test with volatiles from unexposed plants. There was no significant difference in preference to volatiles from the commercial hybrid variety WS505 with eggs or without ($\chi^2 = 0.170$, $P = 0.68$) (Fig. S1a). In a dual choice experiment where both plants were exposed to egg deposition, the parasitoids showed a significant preference for the landrace variety C-2101 over the commercial hybrid variety WS505 ($\chi^2 = 8.963$, $P = 0.0028$) (Fig. S1a). *C. sesamiae* was also attracted to odours of uninfested maize plants compared with the blank control, i.e. clean air, in all single choice tests (C-2101: $\chi^2 = 15.87$, $P < 0.0001$; H-2034: $\chi^2 = 7.41$, $P = 0.0065$; WS505: $\chi^2 = 5.66$, $P = 0.0173$) (Fig. S1b) when the choice of infested plant odours was not available.

Systemic effect

Responses of stemborer parasitoids to the headspace samples collected from landrace line C-2101 were tested in the four-arm olfactometer. Systemically collected volatiles were attractive to *T. bournieri* ($F_{1,35} = 9.82$, $P = 0.003$) and *C. sesamiae* ($F_{1,38} = 32.62$, $P < 0.001$) when tested against samples of volatiles from plants without eggs (Fig. 2). This demonstrated that HIPV production by the landraces studied is not limited to the site of egg deposition, but also occurs systemically. In addition, it indicates that the attractive volatiles originate from the plant, rather than from the eggs themselves.

Responses to plants treated with egg material

When maize plants were treated with an ethanolic extract of the adhesive substance underneath *C. partellus* eggs, we again found production of attractive HIPVs. Volatiles from maize plants (landrace line C-2101) treated with the extract were more attractive ($F_{2,34} = 10.39$, $P < 0.001$) to *C. sesamiae* than control plants treated with solvent. (Fig. S5). Similarly, volatiles collected after application of extract to a different leaf on the same plant (i.e. systemic application) were more attractive to *C. sesamiae* than the control plants ($F_{2,34} = 3.49$, $P = 0.029$) (Fig. S5). These results showed that the material coating *C. partellus* eggs, and causing them to adhere to plant leaves, contains an elicitor that induces the emission of HIPVs from
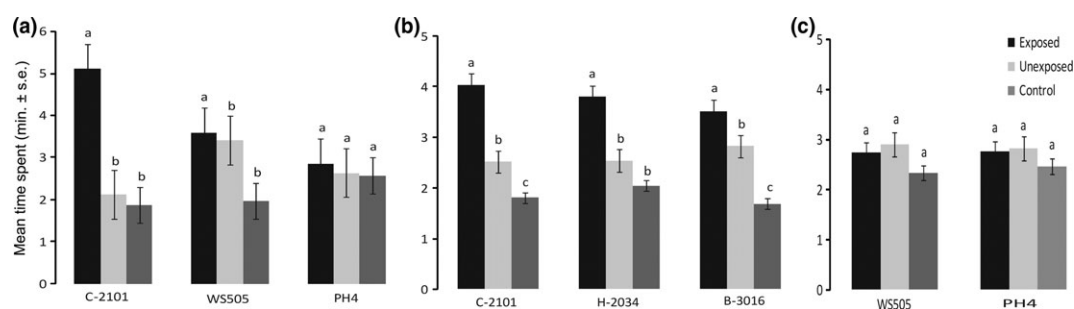

Figure 1 Responses of parasitoids to volatiles collected from maize with or without *C. partellus* eggs in a four-arm olfactometer bioassay. (a) Female *T. bournieri* response to volatiles from C-2101 (Cuba), a maize landrace, and WS-505 (Western Seed Ltd) and PH4 (Kenya Seed Ltd), both of which are commercial maize hybrid varieties. (b) Female *C. sesamiae* response to volatiles from maize landraces C-2101, H-2034 (Haiti) and B-3016 (Brazil). (c) Female *C. sesamiae* response to volatiles from commercial hybrid varieties (WS505 & PH4). Each female parasitoid was observed for 12 min ($n = 12$). Mean (± SE) for time spent (min) in each part of the olfactometer is shown. Parasitoid responses were compared by ANOVA after conversion of the data into proportions and log-ratio transformation. Different letters indicate a significant difference using Fisher's LSD test ($P < 0.05$).
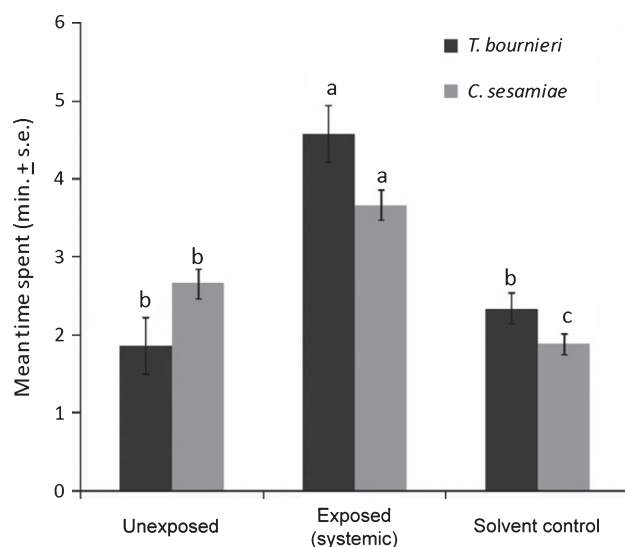
Figure 2 Response of parasitoids to volatiles collected systemically after egg deposition by *C. partellus* on representative maize landrace line C-2101 (Cuba) compared with volatiles from an unexposed plant and solvent control. Each female parasitoid (*T. bournieri* or *C. sesamiae*) was observed in a four-arm olfactometer bioassay for 12 min (*n* = 12). Mean (± SE) for time spent (min) in each part of the olfactometer is shown. Parasitoid responses were compared by ANOVA after conversion of the data into proportions and log-ratio transformation. Different letters indicate a significant difference using Fisher's LSD test (*P* < 0.05).

maize leaves. Although beyond the scope of the current study, identification of the chemical structure of the elicitor in the egg material is an important goal for future research.

Identification of attractive semiochemicals

The GC-EAG recordings with the attractive HIPV samples from landrace lines C-2101, B-3016 and H-2034 revealed that *C. sesamiae* antennae were responsive to (*E*)-ocimene, (*R*)-linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), methyl salicylate, decanal, methyleugenol, (*E*)-(1*R*,9*S*)-caryophyllene, (*E*)-β-farnesene and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (Fig. S2). Electrophysiological activity of these compounds was further confirmed by EAG recordings with authentic compounds (Fig. S3). There was significantly higher emission of (*E*)-(1*R*,9*S*)-caryophyllene ($\chi^2 = 10.42$, $P < 0.0012$), DMNT ($\chi^2 = 8.65$, $P = 0.0033$) and TMTT ($\chi^2 = 4.40$, $P < 0.036$) from the three landrace varieties when bearing *C. partellus* eggs (Figs 3 and 4). Analyses of systemically collected volatiles revealed similar increases in emission of these compounds. Mean emission rates (ng kg^{-1} fresh weight day^{-1}) of EAG active volatile compounds from landraces and commercial maize varieties without and with *C. partellus* eggs are presented in Table 1. Egg deposition by *C. partellus* on the landraces studied was thus found to induce both local and systemic emission of HIPVs which attract key natural enemies of the pest. However, when headspace samples from standard commercial hybrid varieties were analysed, very little difference was found in volatile emissions from exposed and unexposed plants (Fig. S4).

Behavioural response of parasitoids to synthetic compounds

The EAG active HIPVs were tested for attractiveness in the four-arm olfactometer. (*E*)-(1*R*,9*S*)-Caryophyllene, DMNT and TMTT were
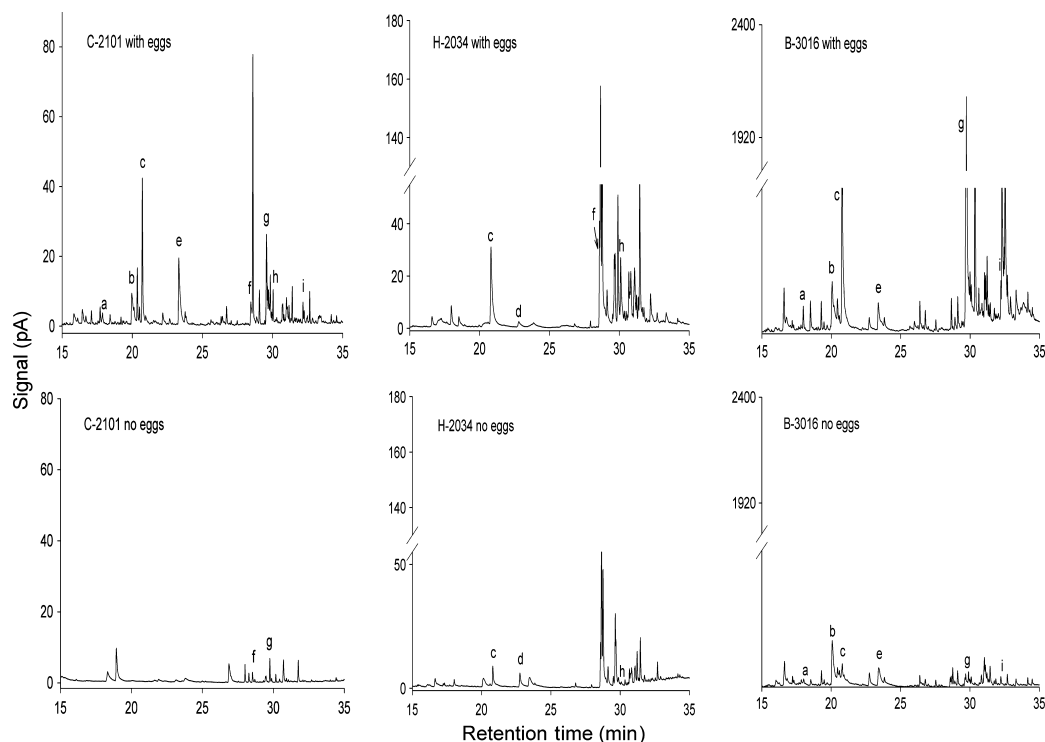

Figure 3 GC profiles of headspace volatiles from maize landraces with and without *C. partellus* eggs, C-2101 (Cuba), B-3016 (Brazil) & H-2034 (Haiti). The identities of EAG active compounds are as follows: (a) (*E*)-ocimene, (b) (*R*)-linalool, (c) (*E*)-4,8-dimethyl-1,3,7, nonatriene (DMNT), (d) methyl salicylate, (e) decanal, (f) methyleugenol, (g) (*E*)-(1*R*,9*S*)-caryophyllene, (h) (*E*)-β-farnesene, (i) (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). Mean abundance (ng kg^{-1} fresh weight day^{-1}) of most EAG active compounds was significantly higher on maize landraces exposed to oviposition by *C. partellus* compared with unexposed controls (*n* = 7; *P* < 0.05).
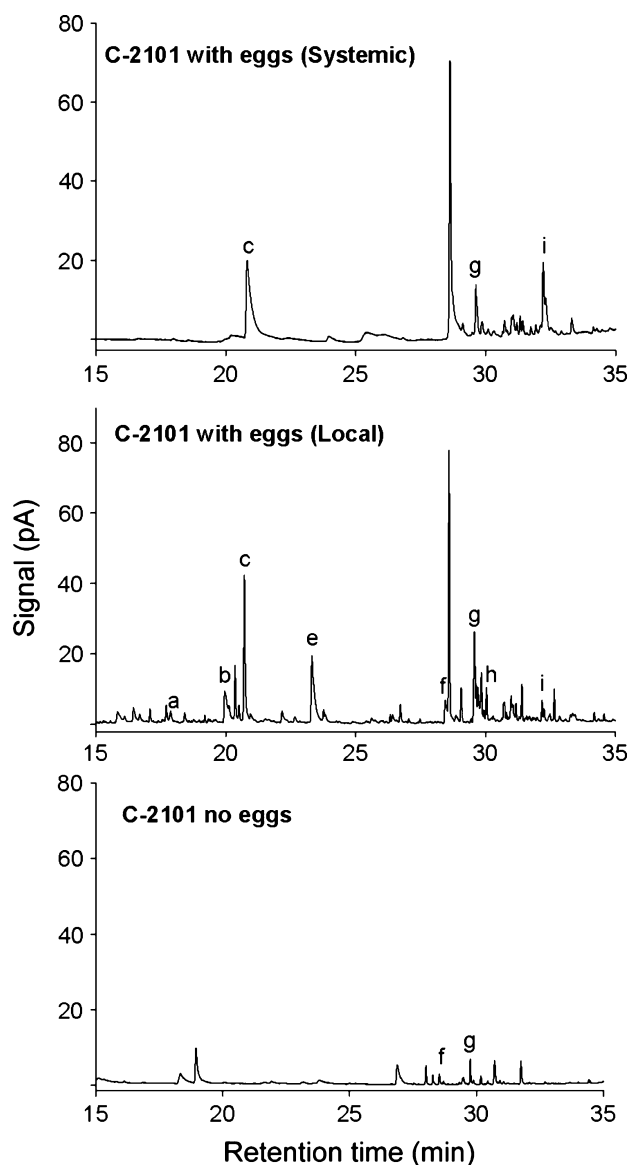
Figure 4 GC profiles of systemically released headspace volatiles from a representative maize landrace line C-2101 (Cuba) with or without *C. partellus* eggs. The identities of EAG active compounds are as follows: (a) (*E*)-ocimene, (b) (*R*)-linalool, (c) (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (d) methyl salicylate, (e) decanal, (f) methyleugenol, (g) (*E*)-(1*R*,9*S*)-caryophyllene, (h) (*E*)-β-farnesene, (i) (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

first tested individually as they were induced by stemborer oviposition at higher levels than the other EAG active compounds. Naïve female egg (Fig. 5a) and larval parasitoids (Fig. 5b) were attracted to a 1 µg dose of the authentic standards of DMNT (*T. bournieri*: $F_{1,35} = 15.89$, $P < 0.001$; *C. sesamiae*: $F_{1,35} = 43.57$, $P < 0.001$) and TMTT (*T. bournieri*: $F_{1,35} = 7.66$, $P = 0.009$; *C. sesamiae*: $F_{1,35} = 45.64$, $P < 0.001$) compared with blank controls, providing further evidence that these compounds play an important role in the host finding behaviour of parasitoids. (*E*)-(1*R*,9*S*)-Caryophyllene was not attractive at this dose (*T. bournieri*: $F_{1,35} = 0.6$; $P = 0.445$; *C. sesamiae*: $F_{1,35} = 0.22$, $P = 0.639$) and a larger dose of 10 µg was required to elicit attraction (*T. bournieri*: $F_{1,35} = 26.63$, $P < 0.001$; *C. sesamiae*: $F_{1,35} = 35.52$, $P < 0.001$).

Attraction of insects to host plant odours involves detection of specific blends of semiochemicals in specific ratios (Bruce *et al.* 2005; Webster *et al.* 2010; Bruce & Pickett 2011). We therefore formulated blends of the synthetic HIPVs at the same concentration and ratio as in one of the attractive headspace samples, C2101 (Cuba) [(*R*)-linalool, DMNT, decanal, methyleugenol, α-copaene, (*E*)-(1*R*,9*S*)-caryophyllene, (*E*)-β-farnesene, pentadecane, and TMTT in a 5.5 : 17.7 : 1.6 : 3.6 : 1.2 : 5.3 : 12.1 : 6.5 : 11.7 ratio]. In bioassays using a 0.5 µg dose, this blend elicited potent attraction of both *T. bournieri* ($F = 12.75$, $P = 0.001$) and *C. sesamiae* ($F_{1,35} = 75.69$, $P < 0.001$) (Fig. 5).

DISCUSSION

Oviposition by herbivorous insects can induce indirect plant defence responses whereby volatiles are emitted that attract egg parasitoids (Hilker & Meiners 2006), although suppression can also occur (Penaflor *et al.* 2011). HIPVs provide parasitoids with early alert cues for plants colonised by their host and thus enhance their foraging efficacy (Colazza *et al.* 2004; Hilker & Meiners 2006; Bruce *et al.* 2010). Plants that are able to produce HIPVs in response to egg deposition have the advantage of defending themselves early on, before hatching larvae can damage the plant. Our study is the first one in which the egg-induced volatile emission effect is shown in maize, an economically important crop plant. However, the effect only occurred in certain landraces and not in the hybrid varieties tested. Three maize landraces, C-2101 (Cuba), B-3016 (Brazil) and H-2034 (Haiti), were found to produce HIPVs attractive to egg (*T. bournieri*) and larval (*C. sesamiae*) parasitoids in response to stemborer (*C. partellus*) egg deposition.

The HIPV emission following oviposition enables egg parasitoids to distinguish odours of plants colonised by hosts. Moreover, the attraction of larval parasitoids in response to oviposition indicates that their recruitment occurs in anticipation of larval hatching and before they damage the plant. Eggs and larvae are both present in fields where maize is attacked by *C. partellus*, a herbivore which has a short life cycle under tropical conditions, with eggs hatching 4 days after laying (Harris 1990). It is therefore highly likely that egg presence also implies larval presence, explaining the observed attraction from the perspective of the larval parasitoid. Being an annual plant with a short life cycle, maize will benefit from recruiting parasitoids even more than perennial plants investigated previously (Hilker & Meiners 2006). Moreover, the maize landrace varieties identified here were capable of attracting not only egg parasitoids, but also larval parasitoids in response to egg deposition. While it is of adaptive value to the plant to emit HIPVs, there is also selection pressure on the parasitoids to respond to such signals, as it enhances their foraging efficiency and thus improves their ecological fitness.

These defensive responses were not exhibited by the commercial hybrid maize varieties tested, suggesting that the ability to emit HIPVs at this early stage of herbivory may have been lost during the breeding process. Previous reports have indicated possible loss of direct defences (Sotelo 1997) and below-ground indirect defences (Rasmann *et al.* 2005; Köllner *et al.* 2008) during breeding and domestication processes. As far as we are aware, this is the first demonstration of an above-ground indirect defence trait, elicited by insect eggs, that is present in landraces, but absent in commercial hybrid maize varieties.

In conclusion, our study demonstrates that oviposition by *C. partellus* induces maize landraces to release volatiles that attract

Table 1 Emission rates (ng kg^{-1} fresh weight day^{-1}) of EAG active volatiles from intact landrace and commercial hybrid maize plants without and with *C. partellus* eggs

| | Mean emission (ng kg^{-1} fresh weight^{-1} day^{-1}) | | | | |
| | Landraces | | Commercial varieties | | |
EAG active compound	Without eggs	With eggs	Without eggs	With eggs	Plants used
(*E*)-Ocimene	1.71 (± 0.65) a	26.96 (± 3.84) b	1.33 (± 0.72) a	5.68 (± 1.04) a	7
(*R*)-Linalool	5.27 (± 1.03) a	28.06 (± 4.59) b	Trace	Trace	7
(*E*)-4, 8-Dimethyl-1,3,7,nonatriene (DMNT)	3.67 (± 0.29) a	145.95 (± 3.29) b	Trace	1.37 (± 0.36)	7
Methyl salicylate	2.11 (± 0.35) a	8.49 (± 0.29) a	Trace	Trace	7
Decanal	4.78 (± 0.14) a	53.96 (± 2.24) b	Trace	Trace	7
Methyleugenol	2.95 (± 0.21) a	31.09 (± 0.25) b	Trace	Trace	7
(*E*)-(1*R, 9S*)-Caryophyllene	3.94 (± 0.15) a	604.32 (± 4.69) b	2.14 (± 0.55) a	2.37 (± 0.84) a	7
(*E*)-β-Farnesene	3.11 (± 0.23) a	49.32 (± 1.84) b	Trace	Trace	7
(*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT)	1.19 (± 0.56) a	61.04 (± 2.47) b	Trace	Trace	7

Means followed by a different letter, within a row, are significantly different (*t*-test, $P < 0.05$). 'Trace' indicates trace amounts, i.e. < 1 ng kg^{-1} freshweight^{-1}day^{-1}.
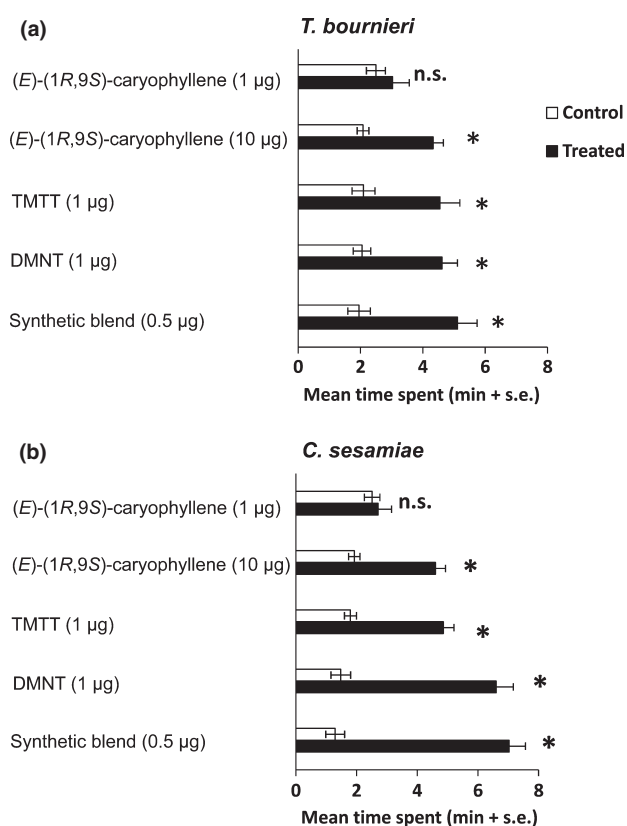

Figure 5 Responses of female parasitoids, (a) *T. bournieri.* (b) *C. sesamiae,* to synthetic standards of landrace volatiles in a four-arm olfactometer bioassay. Each female parasitoid was observed for 12 min ($n = 12$). Twelve female parasitoids were used for each bioassay experiment and each insect was observed for 12 min. Mean (± SE) for time spent (min) in each part of the olfactometer is shown. Parasitoid responses were compared by ANOVA after conversion of the data into proportions and log-ratio transformation. Different letters indicate a significant difference using Fisher's LSD test ($P < 0.05$).

Tumlinson 1992; Zangerl 1999; Dicke & van Loon 2000). This proposed indirect defence strategy, however, appears to have been lost in modern maize hybrids. The current research findings pave the way for developing novel and ecologically sound approaches to the control of destructive stemborer pests by introgression of these traits into mainstream commercial hybrid maize varieties.

AUTHORSHIP

TB, JP, ZK, CM and AT designed the study; AT performed bioassays, volatile collections and gas chromatography analyses; AT and TB analysed the data; CW performed electrophysiological recordings; JC, AT, MB, TB and JP identified volatiles; PM synthesised chemicals; AT, TB, JP and ZK wrote the manuscript; CW, CM, CO and MB contributed to revisions.

egg and larval parasitoids. It also demonstrates that the oviposition associated HIPVs are not only produced at the site of egg deposition (locally), but also systemically throughout the plant. This gives added advantages as it increases the total amount of volatiles released by the plant and hence the strength of the defence signal (Turlings &

REFERENCES

Agelopoulos, N.G., Hooper, A.M., Maniar, S.P., Pickett, J.A. & Wadhams, L.J. (1999). A novel approach for isolation of volatile chemicals released by individual leaves of a plant *in situ. J. Chem. Ecol.*, 25, 1411–1425.

Borlaug, N. (2007). Feeding a hungry world. *Science*, 318, 359.

Bruce, T.J.A. (2010). Tackling the threat to food security caused by crop pests in the new millennium. *Food Security*, 2, 133–141.

Bruce, T.J.A. & Pickett, J.A. (2011). Perception of plant volatile blends by herbivorous insects – finding the right mix. *Phytochemistry*, 72, 1605–1611.

Bruce, T.J.A., Wadhams, L.J. & Woodcock, C.M. (2005). Insect host location: a volatile situation. *Trends Plant Sci.*, 10, 269–274.

Bruce, T.J.A., Midega, C.A.O., Birkett, M.A., Pickett, J.A. & Khan, Z.R. (2010). Is quality more important than quantity? Insect behavioural responses to changes in

a volatile blend after stemborer oviposition on an African grass. *Biol. Lett.*, 6, 314–317.

Chou, T., Tso, H. & Chang, L. (1984). Stereoselective one-step syntheses of trans-ocimene and alpha-farnesene. *J. Chem. Soc. Chem. Commun.*, 20, 1323–1324.

Colazza, S., Fucarino, A., Peri, E., Salerno, G., Conti, E. & Bin, F. (2004). Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids. *J. Exp. Biol.*, 207, 47–53.

Cook, S.M., Khan, Z.R. & Pickett, J.A. (2007). The use of push-pull strategies in integrated pest management. *Annu. Rev. Entomol.*, 52, 375–400.

De Moraes, C.M., Lewis, W.J., Pare, P.W., Alborn, H.T. & Tumlinson, J.H. (1998). Herbivore-infested plants selectively attract parasitoids. *Nature*, 393, 570–573.

De Moraes, C.M., Mescher, M.C. & Tumlinson, J.H. (2001). Caterpillar-induced nocturnal plant volatiles repel nonspecific females. *Nature*, 410, 577–580.

Dicke, M. & van Loon, J.J.A. (2000). Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. *Entomol. Exp. Appl.*, 97, 237–249.

Duffey, S.S. & Stout, M.J. (1996). Antinutritive and toxic components of plant defense against insects. *Arch. Insect Biochem. Physiol.*, 32, 3–37.

Estruch, J.J., Carozzi, N.B., Desai, N., Duck, N.B., Warren, G.W. & Koziel, M.G. (1997). Transgenic plants: an emerging approach to pest control. *Nat. Biotechnol.*, 15, 137–141.

Gaoni, Y. (1977). Lithium aluminium hydride promoted extrusion of SO_2 from sulfolenes and sulfolene-adducts. 1,3-Dienes, 3-chloro- and 3,3-dichloro-1,4-dienes and skipped polyenes. *Tetrahedron Lett.*, 11, 947–950.

Harris, K.M. (1990). Bioecology of *Chilo* species. *Insect Sci. Appl.*, 11, 467–477.

Heil, M. (2008). Indirect defence via tritrophic interactions. *New Phytol.*, 178, 41–61.

Hilker, M. & Meiners, T. (2006). Early herbivore alert: insect eggs induce plant defense. *J. Chem. Ecol.*, 32, 1379–1397.

Howe, G.A. & Jander, G. (2008). Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.*, 59, 41–66.

Kang, S.K., Chung, G.Y. & Lee, D.H. (1987). A convenient synthesis of (*E*)-β-farnesene. *Bull. Korean Soc.*, 8, 351–353.

Kessler, A. & Baldwin, I.T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, 291, 2141–2144.

Kfir, R. (1997). Competitive displacement of *Busseola fusca* (Lepidoptera: Noctuidae) by *Chilo partellus* (Lepidoptera: Pyralidae). *Ann. Entomol. Soc. Am.*, 90, 619–624.

Kfir, R., Overholt, W.A., Khan, Z.R. & Polaszek, A. (2002). Biology and management of economically important lepidopteran cereal stem borers in Africa. *Annu. Rev. Entomol.*, 47, 701–731.

Khan, Z.R., AmpongNyarko, K., Chiliswa, P., Hassanali, A., Kimani, S., Lwande, W. *et al.* (1997). Intercropping increases parasitism of pests. *Nature*, 388, 631–632.

Khan, Z.R., Midega, C.A.O., Bruce, T.J.A., Hooper, A.M. & Pickett, J.A. (2010). Exploiting phytochemicals for developing a 'push – pull' crop protection strategy for cereal farmers in Africa. *J. Exp. Bot.*, 61, 4185–4196.

Köllner, T.G., Schnee, C., Gershenzon, J. & Degenardt, J. (2004). The sesquiterpene hydrocarbons of maize (*Zea mays*) form five groups with distinct developmental and organ-specific distributions. *Phytochemistry*, 65, 1895–1902.

Köllner, T.G., Held, M., Lenk, C., Hiltpold, I., Turlings, T.C.J., Gershenzon, J. *et al.* (2008). A maize (*E*)-β-caryophyllene synthase implicated in indirect defense response against herbivores is not expressed in most American maize varieties. *Plant Cell*, 20, 482–494.

Leopold, E.J. (1990). Elective hydroboration of a 1,3,7-triene: homogeraniol. *Org. Synth.*, 64, 164–171.

Loughrin, J.H, Manukian, A., Heath, R.R. & Tumlinson, J.H. (1995). Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *J. Chem. Ecol.*, 21, 1217–1227.

Maddrell, S.H.P. (1969). Secretion by the Malphigian tubules of *Rhodnius*. The movement of ions and water. *J. Exp. Biol.*, 51, 71–97.

Migui, S.M. & Lamb, R.J. (2003). Patterns of resistance to three cereal aphids among wheats in the genus *Triticum* (Poaceae). *Bull. Entomol. Res.*, 93, 323–333.

Ngi-Song, A.J., Njagi, P.G.N., Torto, B. & Overholt, W.A. (2000). Identification of behaviourally active components from maize volatiles for the stemborer parasitoid *Cotesia flavipes* Cameron (Hymenoptera: Braconidae). *Insect Sci. Appl.*, 20, 181–189.

Ochieng, R.S., Onyango, F.O. & Bungu, M.D.O. (1985). Improvement of techniques for mass-culture of *Chilo partellus* (Swinhoe). *Insect Sci. Appl.*, 6, 425–428.

Overholt, W.A., Ochieng, J.O., Lammers, P. & Ogedah, K. (1994). Rearing and field release methods for *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), a parasitoid of tropical graminaceous stem borers. *Insect Sci. Appl.*, 15, 253–259.

Penaflor, M.F.G.V., Erb, M., Robert, C.A.M., Miranda, L.A., Werneburg, A.G., Dossi, F.C.A. *et al.* (2011). Oviposition by a moth suppresses constitutive and herbivore-induced plant volatiles in maize. *Planta*, 234, 207–215.

Pettersson, J. (1970). An aphid sex attractant 1. Biological studies. *Entomol. Scand.*, 1, 63–73.

Rasmann, S., Köllner, T.G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U. *et al.* (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature*, 434, 732–737.

Sabelis, M.W., Van Baalen, M., Bakker, F.M., Bruin, J., Drukker, B., Egas, M. *et al.* (1999). The evolution of direct and indirect plant defence against herbivorous arthropods. In: *Herbivores Between Plants and Predator*, (eds Olff, H., Brown, V.K. & Drent, R.H.). Blackwell Science Publishers, Oxford, pp. 109–166.

Sotelo, A. (1997) Constituents of wild food plants. In: *Functionality of Food Phytochemicals*. (eds Jones, T. & Romeo, J.J.). Plenum Press, New York, pp. 89–111.

Steinberg, S., Dicke, M., Vet, L.E.M. & Wanningen, R. (1992). Response of braconid *Cotesia* (=*Apanteles*) *glomerata* to volatile infochemicals: effect of bioassay set-up, parasitoid age experience and barometric flux. *Entomol. Exp. Appl.*, 63, 163–175.

Tamiru, A., Getu, E. & Jembere, B. (2007). Role of some ecological factors for an altitudinal expansion of *Chilo partellus* (swinhoe) (Lepidoptera: Crambidae). *SI-NET: Ethio. J. Sci.*, 30, 71–76.

Tamiru, A., Getu, E., Jembere, B. & Bruce, T. (2011). Effects of temperature and relative humidity on the development and fecundity of *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae). *Bull. Entomol. Res.*, DOI: 10.1017/S0007485311000307

Tams, W.H.T. (1932). New species of African Hetrocera. *Entomologist*, 65, 1241–1249.

Tumlinson, J.H., Turlings, T.C.J. & Lewis, W.J. (1993). Semiochemically mediated foraging behavior in beneficial parasitic insects. *Arch. Insect Biochem. Physiol.*, 22, 385–391.

Turlings, T.C.J. & Tumlinson, J.H. (1992). Systemic release of chemical signals by herbivore injured corn. *Proc. Natl Acad. Sci. USA*, 89, 8399–8402.

Turlings, T.C.J., Tumlinson, J.H. & Lewis, W.J. (1990). Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science*, 250, 1251–1253.

Turlings, T.C.J., Bernasconi, M., Bertossa, R., Bigler, F., Caloz, G. & Dorn, S. (1998). The induction of volatile emissions in maize by three herbivore species with different feeding habits: possible consequences for their natural enemies. *Biol. Control*, 11, 122–129.

Wadhams, L.J. (1982). Coupled gas-chromatography – single cell recording – a new technique for use in the analysis of insect pheromones. *Z. Naturforsch.(C)*, 37, 947–952.

Wadhams, L.J. (1990). The use of coupled gas chromatography: electrophysiological techniques in the identification of insect pheromones. In: *Chromatography and Isolation of Insect Hormones and Pheromones* (eds McCaffery, A.R. & Wilson, I.D.). Plenum Press, New York, pp. 281–288.

Webster, B., Bruce, T.J.A., Pickett, J.A. & Hardie, J. (2010). Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. *Anim. Behav.*, 79, 451–457.

Zangerl, A.R. (1999). Locally-induced responses in plants: the ecology and evolution of restrained defense. In: *Induced Plant Defenses against Herbivores and Pathogens: Biochemistry, Ecology, and Agriculture* (eds Agrawal, S. T. & Bent, E.). American Pathological Society Press, St. Paul Minnesota, pp. 231–250.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Responses of *C. sesamiae* to maize odours in a Y-tube olfactometer bioassay. (a) Maize plants with or without *C. partellus* eggs. (b) Uninfested plants versus blank control (clean air). All tests were replicated three times with 20 parasitoids per replicate. Asterisks indicate statistically significant difference using a χ^2 goodness-of-fit

test (*P < 0.05, **P < 0.01, ***P < 0.001, n = 60, ns = not significant, NR = no response).

Figure S2 GC-EAG recordings of responses of *C. sesamiae* antennae to oviposition-induced volatiles from maize landraces C-2101 (Cuba), H-2034 (Haiti) and B-3016 (Brazil). Identified compounds elicited consistent responses from three or more antennae: (a) (E)-ocimene, (b) (R)-linalool, (c) (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (d) methyl salicylate, (e) decanal, (f) methyleugenol, (g) (E)-(1R,9S)-caryophyllene, (h) (E)-β-farnesene, (l) (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). Simultaneous EAG and FID responses were recorded with a customised software program (Syntech, The Netherlands). Upper traces (red): EAG response; lower traces: FID response.

Figure S3 EAG responses of *C. sesamiae* to authentic standards of compounds identified in headspace samples of maize plants exposed to *C. partellus* egg deposition. All compounds were tested at 1 mg mL^{-1} dose and replicated five times. Response data were compared by fitting a linear mixed model with synthetic standards as fixed components and *C. sesamiae* (rep)/authentic standard as the random components. Asterisks indicate a significant difference between mean response to authentic standard and hexane (control) (P < 0.05).

Figure S4 GC profiles of headspace volatiles from standard commercial hybrid varieties (WS505 from Western Seed Ltd and PH4 from Kenya Seed Ltd) with and without *C. partellus* eggs. The identities of EAG active compounds are as follows: (a) (E)-ocimene, (b) (R)-linalool, (c) (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (d)

methyl salicylate, (e) decanal, (f) methyleugenol, (g) (E)-(1R,9S)-caryophyllene, (h) (E)-β-farnesene, (i) (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT).

Figure S5 Response of female *C. sesamiae* to locally and systemically collected volatiles after egg material application on maize landrace line C-2101 (Cuba) in a four-arm olfactometer bioassay. Responses were compared with samples collected from plants treated with ethanol and to a blank solvent. Each parasitoid was observed for 12 min (n = 12). Mean (± SE) for time spent (min) in each part of the olfactometer is shown. Parasitoid responses were compared by ANOVA after conversion of the data into proportions and log-ratio transformation. Different letters indicate a significant difference using Fisher's LSD test (P < 0.05).