



# Contrasting insect attraction and herbivore-induced plant volatile production in maize

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## Abstract

**Main conclusion** The maize inbred line W22 has lower herbivore-induced volatile production than B73 but both fall armyworm larvae and the wasps that parasitize them prefer W22 over B73.

**Abstract** Maize inbred line W22 is an important resource for genetic studies due to the availability of the UniformMu mutant population and a complete genome sequence. In this study, we assessed the suitability of W22 as a model for tritrophic interactions between maize, *Spodoptera frugiperda* (fall armyworm) and the parasitoid wasp *Cotesia marginiventris*. W22 was found to be a good model for studying the interaction as *S. frugiperda* prefers W22 over B73 and a higher parasitism rate by *C. marginiventris* was observed on W22 compared to the inbred line B73. W22 also produced lower amounts of many herbivore-induced volatile terpenes and indole emission upon treatment with *S. frugiperda* oral secretions. We propose that some of the major herbivore-induced terpene volatiles are perhaps impeding *S. frugiperda* and *C. marginiventris* preference and that as yet unidentified compounds are produced at low abundance may be positively impacting these interactions.

**Keywords** Armyworm · Benzoxazinoids · Cotesia · Indole · Terpenes · Volatiles

## Abbreviations

DIMBOA-glc	2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside
DIM2BOA-glc	2-β-D-Glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one
DMNT	3,8-Dimethyl-1,4,7-nonatriene
HBOA-glc	2-β-D-glucopyranosyloxy-1,4-benzoxazin-3-one
HDMBOA-glc	2-Hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside
HMBOA-glc	2-β-D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one
IGL	Indole-3-glycerol phosphate lyase

TMTT	( <i>E,E</i> )-4,8,12-trimethyltrideca-1,3,7,11-tetraene
TPS	Terpene synthase
TRIBOA-glc	2,4,7-Trihydroxy-2H-1,4-benzoxazin-3-(4H)-one-glucoside
TSA1	Tryptophan synthase

## Introduction

Plants defend themselves against insect herbivory using a variety of direct and indirect defense mechanisms. One such indirect defense mechanism involves the attraction of predatory or parasitic insects that can severely impair or remove the herbivores (Degenhardt 2009). For instance, volatile organic compounds produced in the leaves of *Zea mays* (L.) (maize) in response to feeding by the larvae of the lepidopteran *Spodoptera exigua* (Hübner) (beet armyworm) attract the parasitic wasp *Cotesia marginiventris* (Cresson) to oviposit in the larvae (Turlings et al. 1990, 1991). Production of such volatiles, however, can be a double-edged sword, potentially attracting both pest and parasite, as *Spodoptera frugiperda* (J. E. Smith) (fall armyworm) larvae are attracted to volatiles such as linalool produced by damaged

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*Z. mays* (Carroll et al. 2006). Moths of *S. frugiperda* are also attracted to *Z. mays* volatiles, but prefer volatiles from undamaged plants over those from herbivore-induced plants (Signoretti et al. 2012). The moths are electrophysiologically responsive to a range of *Z. mays* volatiles, including linalool, beta-ocimene, alpha-pinene and several short chain alcohols such as hexan-1-ol (Malo et al. 2004). Furthermore, transgenic *Z. mays* lines that constitutively produce the sesquiterpene volatile (*E*)-beta-caryophyllene can be more attractive than both larval and adult female *S. frugiperda* although the volatile in isolation is not attractive (Robert et al. 2013). Such studies support the idea that insects are attracted to bouquets of volatiles rather than individual compounds.

Herbivore-induced volatiles also function in plant–plant communication. For example, indole can prime both systemic tissues and neighboring plants to increase defense responses to subsequent herbivore attack (Erb et al. 2015). Indole also has direct effects on *Spodoptera littoralis*, decreasing food consumption, plant damage and survival of the larvae while increasing larval growth (Veyrat et al. 2016). Both larvae and adults of *S. littoralis* avoid indole-producing plants (Veyrat et al. 2016). Plants can detect an attack by lepidopteran pests via the recognition of fatty acid amide elicitors found in the oral secretions of the insects. In *Z. mays*, application of lepidopteran oral secretions or synthesized fatty acid amides such as volicitin to wounded tissue triggers the production of herbivore-induced volatiles (Alborn et al. 1997). This response is likely mediated by the phytohormone jasmonic acid (Schmelz et al. 2003) and the volatiles produced include indole and a variety of terpenes.

Several studies using a range of approaches including recombinant enzyme assays have identified terpene synthases (TPS) in *Z. mays* that produce sesquiterpenes from farnesyl diphosphate. TPSs include TPS23 (GRMZM2G127336), which produces (*E*)-beta-caryophyllene (Kollner et al. 2008), and TPS10 (GRMZM2G179092), which produces alpha-bergamotene and beta-farnesene (Schnee et al. 2006; Kollner et al. 2009). The homoterpenes 3,8-dimethyl-1,4,7-nonatriene (DMNT), and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) are produced from the sequential action of TPS2 (GRMZM2G046615) and the cytochrome P450s CYP92C5 (GRMZM2G102079) and CYP92C6 (GRMZM2G139467) on farnesyl diphosphate and geranylgeranyl diphosphate, respectively (Richter et al. 2016). TPS2 also produces linalool (Richter et al. 2016), while indole is produced by indole-3-glycerol phosphate lyase (IGL, GRMZM2G046191) (Frey et al. 2000).

Despite numerous studies on the production and function of herbivore-induced plant volatiles in *Z. mays*, many questions remain. The genes that are important for the production of particular volatiles in specific conditions, and the function and importance of specific volatiles within the blend of volatiles are unknown. Furthermore, knowledge gaps exist in

regards to the regulation of herbivore-induced volatiles. One way to fill these knowledge gaps is to compare different *Z. mays* inbred lines. For example, comparison of metabolites and transcriptomes of two *Z. mays* inbred lines has successfully been used to identify candidate genes involved in the response to aphid infestation (Song et al. 2017). However, before such comparisons can be performed effectively, suitable genetic backgrounds for the studies need to be identified.

The *Z. mays* inbred line B73 was the first fully sequenced genome of *Z. mays* (Schnable et al. 2009) and the sequencing and assembly of other *Z. mays* genomes are in progress, including the inbred line W22. This background also boasts a publically available collection of transposon-tagged mutants, known as the UniformMu population, which allows for the isolation of mutants in a large number of genes (Settles et al. 2007). Comparisons of B73 and W22 inbred lines have the potential to be useful in both comparative genomics and reverse genetic approaches for elucidating the production and function of herbivore-induced plant volatiles.

In this study, we compared plant–insect interactions and herbivore-induced plant volatile production between B73 and W22 to look for similarities and differences between the two inbred lines, which could be useful for future comparative genomic studies. We also assessed the usefulness of W22 as a model for the functional characterization of herbivore-induced volatiles and benzoxazinoid production in *Z. mays*. From these studies, we discovered that, despite having reduced volatile production, W22 possessed enhanced indirect defense responses and increased attraction for *S. frugiperda* when compared to B73, supporting its utility as a model for studying these tritrophic interactions.

## Materials and methods

### Insect rearing procedures

The *Cotesia marginiventris* colony was isolated from Belle Glade in Florida, USA, and maintained in an environmentally-controlled chamber at 21–23 °C, 70–75% relative humidity and a photoperiod of 13:11 (light:dark). Adults are housed in a rearing container (plastic frame with organdy cloth) and provided honey, which was smeared onto the walls of the container. Additionally, the cage contained a cotton ball with a 10% sugar solution (v/v) in a 59.2-mL deli cup and 4–5 Kimwipes® EK-L to provide resting sites for the adult wasps.

Two to three days after eclosion of the adult wasps, *S. frugiperda* larvae that were 4–5 days old were provided for oviposition. Using the top half of a Petri dish a Bounty® white paper towel was folded in half, cut to the dimension of the Petri dish, and moistened with water. Fifteen to 20 *Z. mays* leaf pieces (5.1 cm) were then

placed on the moist toweling and the *S. frugiperda* larvae placed on the leaves. Up to 100 larvae are presented to the adults at one time. The larvae were left in the cage for approximately 48 h. Then larvae were removed from the cage placed individually in 32-well rearing trays with general lepidopteran artificial diet (Southland Products Inc.). Parasitoid cocoons began to form 10 days post exposure and adult wasps emerged 4–5 days later.

The *S. frugiperda* colony was initially obtained from Benzon Research Inc. (Carlisle, PA, USA) and was maintained as described in Hay-Roe et al. (2016) but reared on general lepidopteran artificial diet rather than pinto bean diet.

### Insect bioassays

For feeding choice assays, one leaf section measuring 8 cm<sup>2</sup> was taken from the 4th leaf each of a two-week-old W22 and B73 plants, and placed on opposite sides of a Petri dish. This was repeated for a total of 30 Petri dishes. A single first instar *S. frugiperda* was placed in the center of each Petri dish equidistant from the two leaf sections. The Petri dishes were sealed with Parafilm<sup>®</sup> and alternately orientated to negate any effects of ambient light on larval choice. Feeding choice was scored as the presence of the larvae on the W22 or B73 leaf section for each of the 30 Petri dishes. If larvae were not on a leaf section it was scored as such and excluded for that time point. Scoring was undertaken immediately after setup and 6, 24 and 30 h after setup.

Larval growth assays were conducted by placing a pre-weighed first instar *S. frugiperda* on each of twenty, 2-week-old W22 or B73 plants that were housed in separate cages. Larvae were weighed 3–6 days after exposure.

Parasitism assays with *C. marginiventris* were performed by placing eight first instar *S. frugiperda* per plant on 2-week-old B73 and W22 plants. Four plants from each line were then placed in a randomized arrangement in an insect cage with no contact between plants in different pots. Four such cages were setup per experiment all containing both B73 and W22 plants. At 8 a.m. 2 days later, 20 mated *C. marginiventris* wasps were released into each cage. At 1 p.m., the plants were removed from the cages and the *S. frugiperda* larvae separated into individual wells of sealed rearing trays containing general lepidopteran diet. Equal numbers of larvae were removed from B73 and W22 plants in each cage. The *S. frugiperda* larvae were assessed for the presence of *C. marginiventris* larvae by scoring for the emergence of *C. marginiventris*. The percentage of *S. frugiperda* collected from B73 or W22 plants parasitized by *C. marginiventris* was determined per cage.

### Jasmonic acid and volatile analysis

To induce jasmonic acid and volatile production, 2-week-old B73 and W22 plants were mechanically wounded across a square centimeter area with a razorblade on the 4th leaf (wound-only controls) or wounded and treated with 7  $\mu$ L of *S. frugiperda* oral secretions. Oral secretions were collected from *S. frugiperda* larvae that had been feeding on a mix of B73 and W22 leaves for 3 days. For determination of jasmonic acid levels, a 6-cm<sup>2</sup> section of the leaf encompassing the treatment site was excised at 0, 1, 2 and 3 h after treatment and flash frozen in liquid nitrogen. Jasmonic acid was then methylated, isolated by vapor-phase extraction and analyzed by chemical ionization gas chromatography–mass spectrometry as described in (Schmelz et al. 2004). For measurement of volatiles, plants were placed in volatile collection chambers immediately after treatment. Headspace volatiles were collected either for 16 h total or for 4-h periods over a 16-h time course and volatiles were measured by gas chromatography with flame ionization detection as described previously (Block et al. 2017).

### Benzoxazinoid analysis

Benzoxazinoids [2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-glc), 6-methoxy-benzoxazolin-2-one (MBOA), 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA), and 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-glc), 2- $\beta$ -D-glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA-glc), 2- $\beta$ -D-glucopyranosyloxy-1,4-benzoxazin-3-one (HBOA-glc), 2- $\beta$ -D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-glc), 2- $\beta$ -D-glucopyranosyl-oxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one (HDM2BOA-glc), (2- $\beta$ -D-glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one (DIM2BOA-glc) and 2,4,7-trihydroxy-2H-1,4-benzoxazin-3-(4H)-one-glc (TRIBOA-glc)] were analyzed in leaf tissue from 2-week-old B73 and W22 plants wounded across a square centimeter area with a razorblade on the 4th leaf and treated with 7  $\mu$ L of *S. frugiperda* oral secretions. Tissue including and surrounding the wound site was collected at 0, 4, 8 and 16 h after treatment. Samples of approximately 100 mg were ground for 30 s in a Mini-beadbeater (Biospec Products) with 1 g of “Zirmil Y” 1.25-mm ceramic beads (Saint-Gobain Zirpro) in the presence of 1 mL extraction buffer (98% methanol (v/v), 2% glacial acetic acid (v/v), including 5  $\mu$ g mL<sup>-1</sup> 2-benzoxazolinone (Sigma-Aldrich) as an surrogate compound), sonicated for 20 min, and centrifuged at 16,000g for 10 min. Supernatant was filtered through a 0.22- $\mu$ m PVDF membrane (Millipore Sigma) before analysis.

Samples were injected (1  $\mu\text{L}$ ) onto an Agilent 6550 ultra-performance liquid chromatography coupled to a hybrid quadrupole orthogonal time of flight mass spectrometer (UPLC-QTOF; Santa Clara, CA, USA) equipped with a dual Agilent Jet Stream electrospray ionization source. All samples and standards were analyzed in both positive and negative ionization modes. Mobile phases consisted of 0.1% formic acid (v/v) in A: water and B: acetonitrile. Separation was achieved at 0.4  $\text{mL min}^{-1}$  flow rate with a ZORBAX Eclipse Plus C18 RRHD (50 $\times$ 2.1 mm i.d., 1.8  $\mu\text{m}$  particle size, Agilent Technologies) with corresponding guard column held at 30  $^{\circ}\text{C}$  and a binary gradient elution program as follows: initial conditions, 5% B; 2 min hold time, linear gradient to 100% B in 20 min; hold at 100% B for 2 min (24 min total run time); 7 min post-run period to re-equilibrate column to initial conditions. The QTOF was operated in MS scan mode (70–2000  $m/z$ ) at 3 spectra  $s^{-1}$  with the following source conditions: capillary voltage, 3000 V; nozzle voltage, 2000 V; gas temperature, 200  $^{\circ}\text{C}$ ; gas flow, 13  $\text{L min}^{-1}$ ; nebulizer pressure, 20 psig; sheath gas flow, 12  $\text{L min}^{-1}$ . A reference mass solution was continuously introduced to the source to verify mass accuracy during all analyses.

Collected data were mined for tentative benzoxazinoids previously identified in *Z. mays* using Agilent MassHunter Qualitative Analysis (B.07.00) found by formula algorithm. This feature uses input formulae to calculate mono-isotopic molecular mass and then isolates candidate matches based on isotope spacing and abundance patterns. Candidate chemical formulae were compiled from previously identified *Z. mays* metabolites (Glauser et al. 2011; Marti et al. 2013). Tentative identifications were further supported by comparison with reported MS ionization, MS/MS fragmentation patterns and relative retention times of purified benzoxazinoids in maize (Glauser et al. 2011; Marti et al. 2013) and are listed in Suppl. Table S1. MS/MS data were collected at three collision energies (10, 20 and 40 V).

Neat, purified HDMBOA-glc standard was suspended in methanol to prepare calibration standards (0.237–80.9  $\mu\text{g mL}^{-1}$ ). The HDMBOA-glc standard curve was used to quantitate all putative metabolites, as benzoxazinoid glycosides reportedly have similar MS response (Glauser et al. 2011). Monitored quantitation and qualifications are reported in Suppl. Table S1.

### Gene expression analysis

Gene expression was induced on 2-week-old B73 or W22 plants by wounding a square centimeter area with a razorblade on the 4th leaf and treating with 7  $\mu\text{L}$  of *S. frugiperda* oral secretions. Tissue was collected at 0, 2, 4 and 6 h after treatment. Extraction of RNA and first-strand cDNA synthesis was conducted using Direct-Zol RNA miniprep kit (Zymo Research) and Superscript IV First

Strand Synthesis System (Invitrogen) according to manufacturer's directions. Quantitative RT-PCR analyses were done using a Bio-Rad CFX96 Real-Time System with a C1000 Touch Thermal Cycler (Bio-Rad Laboratories). Six biological replicates were analyzed with three technical replicates for each. A given reaction included 10  $\mu\text{L}$  of SsoAdvanced Universal SYBR Green Supemix (Bio-Rad Laboratories, Cat. 172-5274), 100 nM of each gene-specific primer, and 1  $\mu\text{L}$  of cDNA (diluted 20-fold from cDNA reaction) in a final volume of 20  $\mu\text{L}$ . The relative abundance of transcripts was normalized to the folylpolyglutamate synthase (FPGS) reference control gene, which was amplified using primers tested and developed as previously described (Manoli et al. 2012). Relative quantities were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen 2001). The following gene-specific primer pairs were used GRMZM2G046191 (Iglfw and Iglrev); GRMZM2G085381 (Bx1fw and Bx1rev); GRMZM2G085661 (Bx2fw and Bx2-rev); GRMZM2G063756 (Bx5fw and Bx5rev); GRMZM6G617209 (Bx6fw and Bx6rev); GRMZM5G841619 (Tsa1fw and Tsa1rev); GRMZM2G046615 (Tps2fw and Tps2rev); GRMZM2G179092 (Tps10fw and Tps10rev) as in (Huffaker et al. 2013). Primer sequences are detailed in Suppl. Table S2.

### Statistical analysis of results

Statistical analysis was conducted using paired *t* tests for pairwise analysis and ANOVA for multi-treatment analysis. Results were considered statistically significant with  $P \leq 0.05$ .

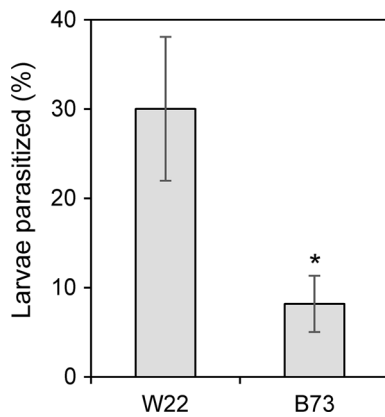
## Results

### Inbred line W22 has increased indirect defenses and attractiveness to *S. frugiperda* in comparison with B73

To determine whether the indirect defense responses of *Z. mays* differ between the two inbred lines, the preference of *C. marginiventris* for parasitism of larval *S. frugiperda* on infested B73 or W22 plants was assessed (Fig. 1). When provided the choice between *S. frugiperda* infested B73 or W22 plants, *C. marginiventris* located and oviposited in 30% of the *S. frugiperda* larvae on W22 plants compared to 7% of the larvae on B73 plants within the time frame assessed. This higher rate of parasitism by *C. marginiventris* on W22 plants compared to B73 plants suggests that *S. frugiperda* are more easily located on W22 plants.

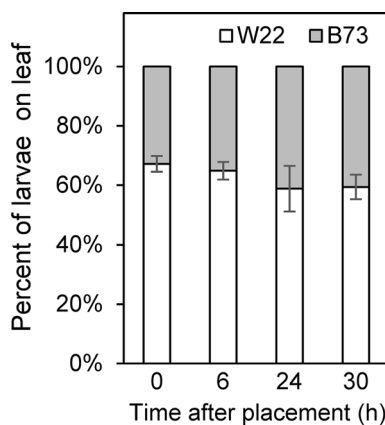
To test if the enhanced preference of *C. marginiventris* for W22 compared to B73 plants is also reflected in a greater





**Fig. 1** *Cotesia marginiventris* parasitize a higher proportion of *S. frugiperda* larvae on W22 than those on B73. Larvae of *S. frugiperda* were placed on B73 and W22 plants and *C. marginiventris* released into the cages. *S. frugiperda* were scored for parasitism. Bars are  $\pm$  standard error,  $n=4$ ; \*significantly different to W22;  $P<0.05$  by paired  $t$  test

preference of *S. frugiperda* larvae, the preference of *S. frugiperda* for B73 versus W22 was examined using a feeding choice assay (Fig. 2). Larvae of *S. frugiperda* showed a strong preference for W22 over B73 leaf sections, with 65% of the larvae initially moving towards W22 leaf sections and only 35% initially moving towards B73 leaf sections. This preference was maintained throughout the 30-h time course with a final selection of 60% of the larvae choosing to feed on W22 and 40% on B73 leaf sections. These data show that *S. frugiperda* prefers to feed on W22 leaves rather than on B73. Therefore, both *C. marginiventris* and *S. frugiperda* have an enhanced preference for W22 compared to B73.

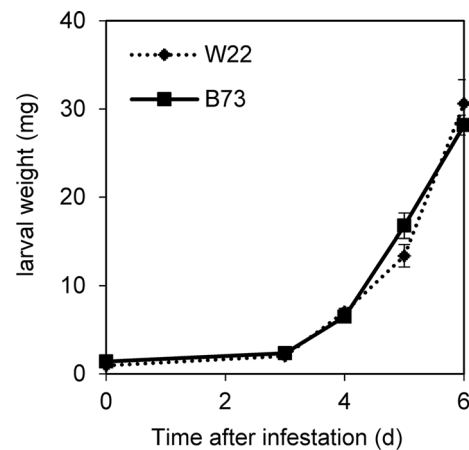


**Fig. 2** Larvae of *S. frugiperda* prefer W22 over B73 in feeding choice assay. Larval *S. frugiperda* were placed equidistant from B73 and W22 leaf pieces. Each larvae was scored for initial choice (0 h) and feeding choice at specified times after initial placement. Graph shows percent of larvae on B73 and W22 leaves over time. Bars are  $\pm$  standard error,  $n=4$

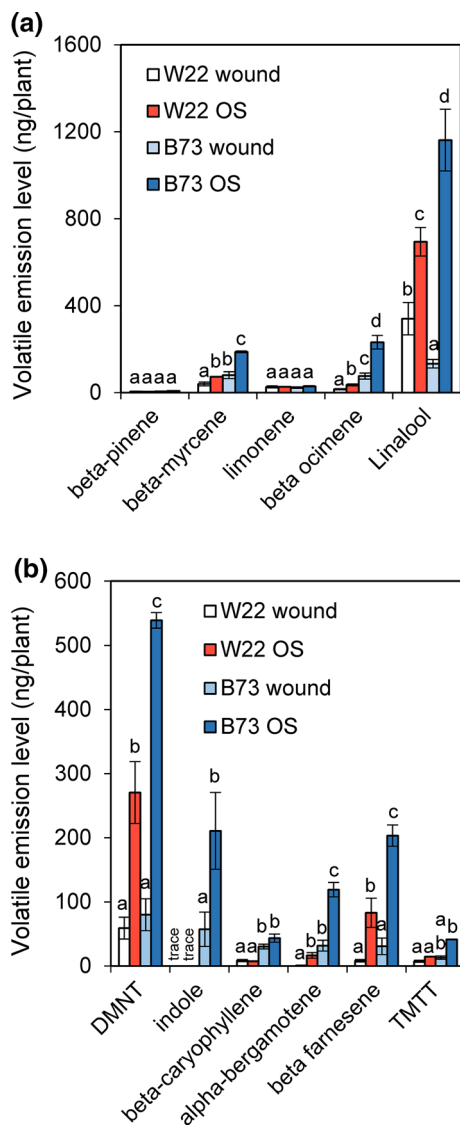
To determine if the feeding preference of *S. frugiperda* for W22 was due to factors from B73 that could negatively impact *S. frugiperda* growth, a larval growth assay was performed on B73 and W22 plants (Fig. 3). No significant difference in larval growth was seen between larvae grown on B73 and W22 over the 6-day assay period. These data suggest that the differences observed during the feeding choice assay were not due to differences in toxicity or nutritional value between the two lines.

**W22 has reduced herbivore-induced volatile production compared to B73**

Both *S. frugiperda* and *C. marginiventris* preferred W22 over B73. Differences in insect preference to host plants are thought to reflect different volatile profiles. To determine if this was the case for B73 and W22, the production of the major wound and herbivore-induced volatiles was examined in the two lines (Fig. 4). The treatment of wounded *Z. mays* leaves with oral secretions from *S. frugiperda* leads to the emission of herbivore-induced plant volatiles. These volatiles include several monoterpenes, sesquiterpenes, homoterpenes and indole. Comparative analysis of monoterpene emission from B73 and W22 plants after a 16-h collection period revealed that the monoterpenes beta-pinene and limonene, which were not induced by oral secretions when compared to wound-only controls, were produced in similar amounts in B73 and W22 (Fig. 4a). In contrast, the monoterpenes beta-myrcene and beta-ocimene, which were induced by oral secretions, were induced to higher levels in B73 than in W22. These monoterpenes were also emitted at higher levels in B73 in the wound-alone controls. The terpene alcohol linalool was induced by oral secretions to



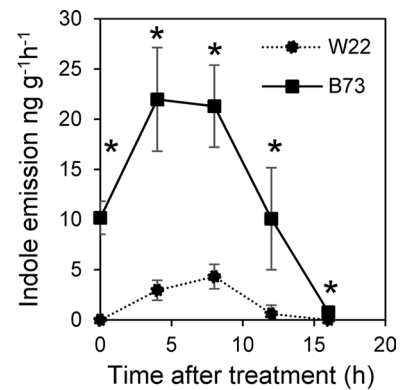
**Fig. 3** Larval growth of *S. frugiperda* is comparable on W22 and B73 plants. Larvae of *S. frugiperda* were placed on B73 and W22 plants and larval growth measured over 6 days. Bars are  $\pm$  standard error,  $n=20$



**Fig. 4** *Spodoptera frugiperda* oral secretion-induced volatile emission levels in W22 and B73. W22 and B73 plants were treated with *S. frugiperda* larval oral secretions (OS) or a wound-only control. Head-space volatiles were collected over 16 h after treatment and quantified. These volatiles included monoterpenes (a) and sesquiterpenes, homoterpenes and indole (b). Trace, below the level of quantification; bars are  $\pm$  standard error,  $n=6$ ; letters represent significant difference at  $P < 0.05$  by ANOVA

higher levels in B73 than in W22. However, in wounded plants the converse was true, with higher linalool emissions in W22 than B73.

The homoterpenes DMNT and TMTT were also induced by oral secretion treatment in both B73 and W22, with significantly higher induction in B73 (Fig. 4b). There was, however, no significant difference in the wounding-only induction of these homoterpenes between B73 and W22. In regards to the sesquiterpenes, (*E*)-beta-caryophyllene was not induced by oral secretion treatment to levels above that



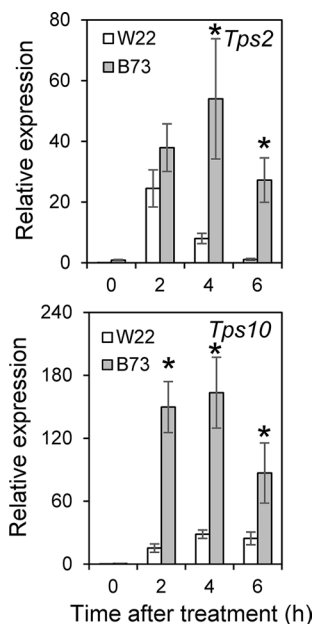
**Fig. 5** Oral secretion-induced indole emission in W22 and B73 Inbred lines. W22 and B73 plants were treated with *S. frugiperda* oral secretions and indole emission was determined using 4 h collection periods for 16 h after treatment. Bars are  $\pm$  standard error,  $n=4$ ; \*significantly different levels between the two lines;  $P < 0.05$  by paired *t* test

of the wound-only control, yet for both treatments it was significantly higher in B73 than in W22. Alpha-bergamotene and beta-farnesene were both induced by oral secretion treatment and induced to a significantly larger extent in B73 than W22. Alpha-bergamotene emission, however, was also significantly higher in B73 than W22 in the wound-alone controls, whereas beta-farnesene emission was not.

Only trace levels of indole were found in W22 in either the oral secretion or wounding treatments after 16 h of collection. B73 produced detectable levels of indole in response to both treatments, as well as higher levels in response to oral secretion than to wounding alone (Fig. 4b). A more detailed analysis of indole production involving collection of emitted volatiles for 4-h periods over a 16-h time course after treatment with oral secretion revealed that W22 produced low levels of indole around 4–8 h after treatment (Fig. 5). B73 showed similar kinetics of indole production but at least fourfold higher emission rates than B73. These data show a general trend of a stronger induction of terpene volatiles and indole production in response to oral secretions in B73 than in W22.

### W22 has reduced induction of terpene synthase gene expression

To assess whether differential expression of terpene synthase genes was a possible explanation for the observed differences in terpene volatile production between B73 and W22, the herbivore-induced expression of two terpene synthases was assessed (Fig. 6). Expression of *Tps2*, which is involved in the production of linalool, DMNT and TMTT, was strongly induced in response to *S. frugiperda* oral secretions in both B73 and W22 by 2 h after treatment. However by 4 h after treatment expression of *Tps2* in B73 was still

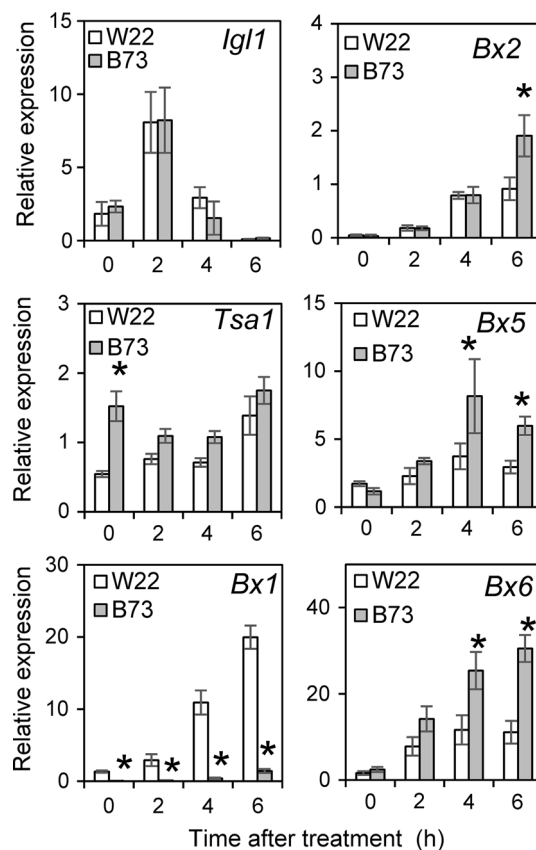


**Fig. 6** Oral secretion-induced terpene synthase expression. B73 and W22 inbred lines were treated with oral secretions from *S. frugiperda* larvae and expression of the terpene synthases *Tps2* and *Tps10* determined over time. Bars are  $\pm$  standard error,  $n=6$ ; \*significantly different to W22;  $P<0.05$  by paired  $t$  test

increasing and remained high at 6 h after treatment, while in W22, it steadily decreased and had returned to basal levels by 6 h after treatment. Expression of *Tps10*, which is involved in the production of alpha-bergamotene and beta-farnesene, was induced to higher levels in B73 than in W22 by oral secretion treatment, though the kinetics of induction were comparable. These data show that the reduced production of these volatiles in W22 compared to B73 correlates with reduced expression of the enzymes involved in their synthesis.

**W22 has comparable *Igl* expression but increased *Bx1* expression compared to B73**

To determine if the reduction of oral secretion-induced indole observed in W22 was due to reduction of expression of the volatile indole biosynthesis gene, *Igl*, the expression of *Igl* was examined in B73 and W22 (Fig. 7). As expected, *Igl* in B73 was rapidly induced by oral secretion treatment, peaking around 2 h after treatment. *Igl* expression in W22 was comparable to that in B73, indicating that differential expression of *Igl* between the two inbred lines was unlikely to be the reason for the differing levels of indole production. IGL from W22 is likely fully functional as comparison of the protein sequences of IGL from B73 and W22 revealed only four amino acids different between the two proteins and



**Fig. 7** Oral secretion-induced expression of indole-3-glycerol phosphate lyases. B73 and W22 inbred lines were treated with oral secretions from *S. frugiperda* and expression of *Igl1*, *Tsa1*, *Bx1*, *Bx2*, *Bx5* and *Bx6* determined over time. Bars are  $\pm$  standard error,  $n=6$ ; \*significantly different to W22;  $P<0.05$  by paired  $t$  test

none of these differences are in the known active sites of the protein (Suppl. Fig. S1).

An alternative hypothesis for the reduced oral secretion-induced indole production in W22 is that another enzyme in W22 is draining indole-3-glycerol phosphate, the substrate for IGL. Two other enzymes in *Z. mays* have been shown to have IGL activity. The first of these is the alpha subunit of tryptophan synthase TSA1 (GRMZM5G841619), which makes indole for the biosynthesis of the amino acid tryptophan (Kriechbaumer et al. 2008). Indole from TSA1 is channeled by the beta subunit of tryptophan synthase and not released into the cell. To determine if differential expression of *Tsa1* was responsible for the reduced indole production in W22, oral secretion-induced *Tsa1* expression levels were determined (Fig. 7). Overall, *Tsa1* expression was not induced in response to oral secretion treatment in W22 or B73 and the two inbred lines produced similar levels of *Tsa1* transcript. These data indicate that TSA1 is unlikely to be responsible for the different indole levels in the two inbred lines.

The other known IGL-like gene in *Z. mays* is *Bx1* (GRMZM2G085381), which makes indole for the production of the herbivore-induced defense metabolites, benzoxazinoids (Frey et al. 1997; Melanson et al. 1997). To determine if differential volatile indole production was associated with differential expression of *Bx1*, the expression of this gene was assessed following oral secretion treatment (Fig. 7). W22 contained 20-fold higher levels of *Bx1* transcript in untreated tissues compared to B73. Higher levels of expression were maintained after treatment, with *Bx1* being strongly induced by oral secretions, while remaining 14- to 25-fold higher in W22 than in B73. To determine if the higher levels of *Bx1* in W22 were due to a general upregulation of benzoxazinoid biosynthesis genes in this inbred, the expression of additional benzoxazinoid genes was examined in the two inbreds (Fig. 7). *Bx2* (GRMZM2G085661), *Bx5* (GRMZM2G063756) and *Bx6* (GRMZM5G841619) were all upregulated in response to oral secretion treatment but unlike *Bx1* their induction was higher in B73 than in W22.

### Induced production of benzoxazinoids in B73 and W22

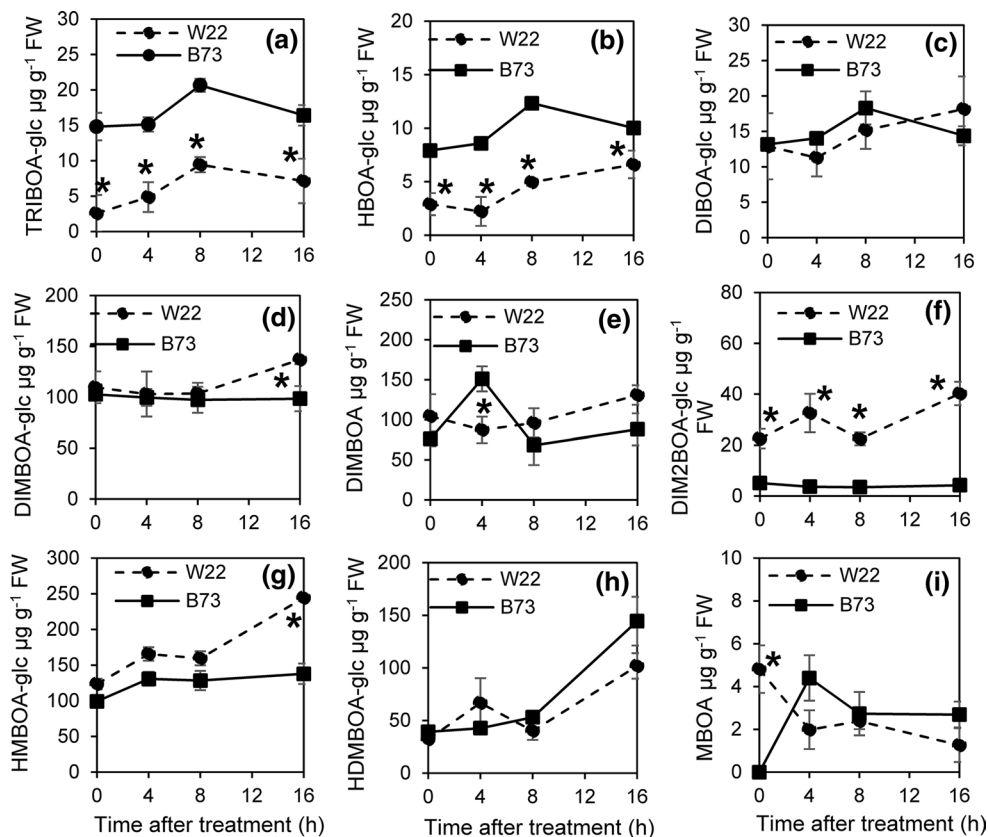
To determine if observed differences in Bx gene induction between B73 and W22 correlated with changes in production of benzoxazinoids, production of a range of benzoxazinoid

was measured at 4, 8 and 16 h after treatment with oral secretions from *S. frugiperda* (Fig. 8). Levels of several benzoxazinoids were significantly different between B73 and W22 plants. B73 had higher levels of TRIBOA-glc and HBOA-glc than W22 in both induced and un-induced tissues. W22 had elevated levels of DIM2BOA-glc compared to B73. HMBOA-glc and DIMBOA-glc had enhanced accumulation in W22 at 16 h after treatment that did not occur in B73, while the activated derivative DIMBOA displayed a peak at 4 h after treatment in B73 that did not occur in W22. However, not all benzoxazinoids were different as B73 and W22 displayed comparable levels of DIBOA-glc and HDMBOA-glc.

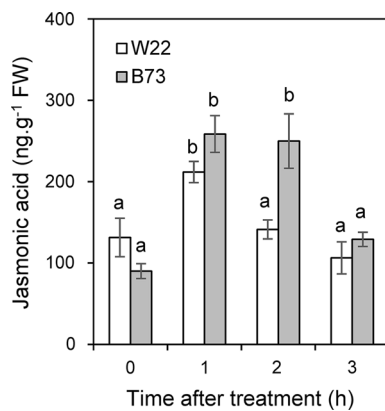
### Induced production of jasmonic acid in B73 and W22

The phytohormone jasmonic acid is a known regulator of the response of *Z. mays* to insect infestation, including regulating the production of herbivore-induced volatiles. The induction of jasmonic acid in response to treatment with oral secretions from *S. frugiperda* was, therefore, compared in the two lines (Fig. 9). Both B73 and W22 showed a strong induction of jasmonic acid production 1 h after treatment, with levels in B73 not statistically different from those of W22. However, the induction of jasmonic acid was maintained in B73 for 2 h after treatment, returning to

**Fig. 8** Oral secretion-induced benzoxazinoids production in W22 and B73 Inbred lines. W22 and B73 plants were treated with *S. frugiperda* oral secretions and benzoxazinoid levels determined for 16 h after treatment. TRIBOA-glc (a), HBOA-glc (b), 2- $\beta$ -D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one (DIBOA-glc (c); DIMBOA-glc (d), DIMBOA (e), DIM2BOA-glc (f), HMBOA-glc (g), HDMBOA-glc (h) and 6-methoxybenzoxazolin-2-one (MBOA, i). Bars are  $\pm$  standard error,  $n = 4$ ; \*significantly different levels between the two lines;  $P < 0.05$  by ANOVA







**Fig. 9** Oral secretion-induced jasmonic acid production in W22 and B73 inbred lines. W22 and B73 plants were treated with *S. frugiperda* oral secretions and jasmonic acid levels determined. Bars are  $\pm$  standard error,  $n=4$ ; letters represent significant difference at  $P<0.05$  by ANOVA

pre-treatment levels only after 3 h, while in W22 jasmonic acid levels returned to pre-treatment levels by 2 h post treatment. This led B73 to contain higher levels of jasmonic acid than W22 2 h after treatment with *S. frugiperda* oral secretions. These data show that B73 has a more sustained jasmonic acid induction than that of W22.

## Discussion

Our study comparing the two *Z. mays* inbred lines (B73 and W22) shows that herbivore *S. frugiperda* prefers W22 over B73 in feeding choice assays. This is true for both initial choice and choice after feeding, and this initial choice suggests that W22 is more attractive to the larvae. However, the choice to remain on W22 may also be due to differences in arrestment, host acceptance factors and feeding preferences. The comparable growth of the larvae on W22 and B73 plants suggests that the observed preference is not due to significantly different levels of antifeedant or antinutritive compounds. Our data also show that the parasitoid *C. marginiventris* has a higher successful parasitism rate in *S. frugiperda* larvae feeding on intact W22 plants when given a direct choice between larvae on W22 and B73 plants. This phenotype could be due to an increased ability of *C. marginiventris* to locate larvae on W22, increased arrestment on W22, increased oviposition preference or lower resistance of the larvae feeding on W22 to parasitism.

Levels of the regulatory phytohormone jasmonic acid are also partially limited in W22, which would suggest it has a weaker defense response to *S. frugiperda* than B73. W22 also has lower levels of several herbivore-inducible volatiles. Our data show that the reduced indole production in W22 was likely not due to a loss of *Igl* expression. The enhanced

expression of *Bx1* in W22 initially indicated that reduced indole may be due to the intersection of the pathways for the production of volatile indole and the production of benzoxazinoids due to siphoning of the indole precursor, indole-3-glycerol phosphate into the benzoxazinoid pathway. However, analysis of additional Bx genes showed that *Bx2*, *Bx5* and *Bx6* were all more induced in B73 than W22 suggesting that there is unlikely to be a higher flux to benzoxazinoids in W22. Measurement of individual benzoxazinoids revealed that several compounds at early stages in the pathway such as TRIBOA-glc (the product of *Bx6*) and HBOA-glc were elevated in B73 compared to W22, though the levels remained relatively low. Later, more abundant products such as DIM2BOA-glc, DIMBOA-glc and HMBOA-glc were all elevated in W22 compared to B73. These data showed differential benzoxazinoid regulation and profiles between the two inbreds but did not point to an extreme increased flux in W22 that could account for the reduced indole emission. These different benzoxazinoid profiles did not seem to differentially impact the growth of *S. frugiperda* larvae despite the known differences in toxicity of the different compounds. An alternate hypotheses for the disconnect between the relative expression of *Igl1* and *Bx1* and the production of volatile indole and benzoxazinoids include, post-transcriptional regulation of protein accumulation and/or enzyme function, and differences in the availability of the common precursor in both pathways (indole-3-glycerol phosphate).

Indole plays an important role in priming both neighboring plants and systemic tissues to produce higher levels of herbivore-induced terpene volatiles (Erb et al. 2015). However, loss of *Igl* gene function abolishes the production of herbivore-induced indole without impacting the production of other herbivore-induced volatiles in naïve plants (Erb et al. 2015). It is, therefore, unlikely that the reduced indole production itself is responsible for the reduced level of herbivore-induced terpenes that was observed in W22. Reduced volatile induction may be partially due to the less-sustained induction of jasmonic acid in W22 plants. This is likely to be the case for homoterpene production, as changes in *Tps2* expression mirror those seen for jasmonic acid. However, based on the results for other terpenes, such as those produced by TPS10, it is likely that other factors involved in regulating gene expression also differ between the two inbreds.

The terpene alcohol linalool was previously shown to be an attractant for *S. frugiperda* larvae (Carroll et al. 2006). While we observed higher levels of linalool in herbivore-induced B73 plants than in W22 plants, the reverse was true for the wound-only control. This suggests that W22 likely produces more linalool than B73 at the start of our feeding choice assays. This higher linalool could be a determining factor in the initial preference of *S. frugiperda* larvae for W22 over B73. The feeding preference of *S. frugiperda* for

W22 was maintained over several days of feeding, indicating that factors other than linalool production were also involved.

*Zea mays* inbred lines have a wide variety of amounts and types of herbivore-induced volatiles (Degen et al. 2004). Field studies using six maize inbred lines chosen for variation in their herbivore-induced volatile production and infested with *S. frugiperda* showed that variation in total volatile production did not correlate with variation in parasitism rates by *C. marginiventris* (Degen et al. 2012). Furthermore, treatment of *Z. mays* with the resistance elicitor benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid *S*-methyl ester or the plant strengthener laminarin led to the reduced production of the herbivore-induced volatiles indole, (*E*)-beta-caryophyllene, beta-bergamotene, and beta-farnesene (Sobhy et al. 2012). These treated plants were more attractive to the parasitic wasps, *C. marginiventris*, *Camponotus sonorensis*, and *Microplitis rufiventris* (Kokuiev) (Sobhy et al. 2012). It is not, therefore, necessarily surprising that, despite the reduced levels of major volatiles, W22 was more attractive to *C. marginiventris*.

Some sesquiterpene volatiles from *Z. mays* are involved in insect attraction. For instance, (*E*)-beta caryophyllene produced by roots upon attack by the coleopteran pest *Diabrotica virgifera virgifera* (LeConte) attracts the entomopathogenic nematodes, which kill the beetles. *Z. mays* plants that do not produce (*E*)-beta-caryophyllene had a fivefold lower infection rate of the beetles with entomopathogenic nematodes than was found in wild-type plants (Rasmann et al. 2005). *C. marginiventris* can also learn to be attracted to (*E*)-beta caryophyllene (Kollner et al. 2008). Experienced wasps are more strongly attracted to a specific blend of volatiles after they have perceived the blend during successful oviposition, indicating that many attraction signals are learned (D'Alessandro and Turlings 2005).

However, some compounds emitted by infested *Z. mays* may be repellent to or mask attractiveness to *C. marginiventris* (D'Alessandro et al. 2009). This phenomenon has been observed for naïve females of the parasitoid *M. rufiventris*, which preferred volatile blends without indole, over those with indole, indicating that indole was masking the attractiveness of the other volatiles (D'Alessandro et al. 2006). This study, however, also showed that indole was not masking the attractiveness of volatile blends for *C. marginiventris* (D'Alessandro et al. 2006). It may be possible that some of the terpenes produced at higher levels in B73 than W22 may be masking less-abundant attractants, or may be actively deterring *S. frugiperda* and *C. marginiventris*.

A strong possibility also exists that the major determinants of attraction are not the major herbivore-induced volatiles that we measured in our assays. Indeed, olfactometer assays with naïve *C. marginiventris* demonstrated that they are likely attracted to polar volatiles, which are produced

at low levels by infested plants (D'Alessandro et al. 2009). While the identity of these compounds remains undetermined, our study indicated that the inbred line W22 may be a good model for genetically elucidating low-abundant compounds as indicated by the higher attractiveness and/or less-repellent volatile profile of the inbred line. The W22 full genome sequence, coupled with the availability of mutants in the W22 background should aid in the elucidation of the last pieces of the puzzle of how volatiles influence this tri-trophic interaction.

**Author contribution statement** AB, CH and SC conceived and designed research, AB, CH, CR and SC conducted experiments, CR and RM contributed new analytical tools, AB, CH and CR analyzed data, AB wrote the manuscript with contributions from all authors.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflicts of interest with this study. The use of trade name, commercial product or corporation in this publication is for the information and convenience of the reader and does not imply an official recommendation, endorsement or approval by the US Department of Agriculture or the Agricultural Research Service for any product or service to the exclusion of others that may be suitable. USDA is an equal opportunity provider and employer.

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