

Effects of Genetic Modification on Herbivore-Induced Volatiles from Maize

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Abstract Large-scale implementation of transgenic crop varieties raises concerns about possible nontarget effects on other organisms. This study examines the effects of genetic modification on plant volatile production and its potential impact on arthropod population dynamics. We compared herbivore-induced volatile emissions from *Bacillus thuringiensis* Berliner (Bt) maize plants to those from a non-transformed isolate following exposure to various types of leaf damage. When equal numbers of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) larvae fed on Bt and non-Bt maize, volatile emissions were significantly lower in the transgenic plants, which also exhibited less leaf damage. When damage levels were controlled by adding more larvae to Bt plants, the plants' volatile emissions increased but displayed significant differences from those of nontransgenic plants. Significantly higher amounts of linalool, β -myrcene, and geranyl acetate were released from transgenic maize than from non-Bt plants. Manipulating the duration of feeding by individual larvae to produce similar damage patterns resulted in similar volatile profiles for Bt and non-Bt plants. Controlling damage levels more precisely by mechanically wounding leaves and applying larval regurgitant likewise resulted in similar emission patterns for Bt and non-Bt maize. Overall, changes in the herbivore-induced volatile profiles of Bt maize appeared to be a consequence of altered larval feeding behavior rather than of changes in biochemical plant defense pathways. The implications of these findings for understanding the impacts of plant-mediated cues on pest and natural enemy behavior in transgenic crop systems are discussed.

Keywords Transgenic crops · Bt · Herbivore-induced volatiles · *Zea mays* · Maize · *Helicoverpa zea* · Corn earworm

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Introduction

Genetically modified (GM) crops designed to resist insect herbivores are rapidly being adopted in many parts of the world. Transgenic maize (*Zea mays* L.) engineered to express delta-endotoxins from the soil bacterium *Bacillus thuringiensis* Berliner (Bt) has been commercially available since 1996 and now accounts for 35% of all maize grown in the US (National Agricultural Statistics Service, 2005). Large-scale implementation of transgenic crop technology has sparked substantial debate over the social, economic, and ecological implications of GM agriculture. Concerns about the ecology of insect-resistant transgenic crops have often focused on resistance development by pests and potential negative effects on natural enemies (Gould, 1998; Shelton et al., 2002). These issues are intricately linked to one another because natural enemies can influence the rate that pest populations adapt to resistant plants (Gould et al., 1991). The mechanisms underlying possible unintended ecological effects of transgenic crops warrant increased research attention.

One way in which GM crops might affect pest and natural enemy population dynamics is by altering plant defense mechanisms, including herbivore-induced plant volatiles. Volatile compounds released by plants can influence the behavior of herbivores searching for oviposition sites (Anderson and Alborn, 1999; De Moraes et al., 2001) and foraging natural enemies (Dicke et al., 1990; Dicke and van Loon, 2000; Turlings et al., 1990; De Moraes and Mescher, 2004). The ecological interactions mediated by these induced plant volatiles are often complex and can be quite sophisticated; for example, volatile blends can be keyed to particular herbivore species and attract species-specific parasitoids (De Moraes et al., 1998). Thus, these interactions may be sensitive to any changes in plant biochemistry that alter plant responses.

Volatile responses might be altered in GM crops through unanticipated phenotypic changes in plant defense systems because of pleiotropic effects or insertional mutations caused by incorporating a foreign gene (Schuler et al., 1999a). Significant phenotypic changes induced by genetic modification are not without precedent: the production of lignin, a major structural component of plants, increased in vascular tissues by 33–97% in Bt maize over non-Bt isolines (Saxena and Stotzky, 2001). Similar changes to complex biochemical pathways involved in plant defense might disrupt or alter the ability of plants to recognize and respond to herbivores, leading to significant differences between crop lines.

Induced volatile responses in transgenic crops might also be influenced by changes in interactions between herbivores and plants. The presence of Bt toxin changes the behavior of some insects. Lepidopteran larvae, for example, can detect and avoid plant parts or artificial diet containing varying forms of the toxin (Ashfaq et al., 2001; Gore et al., 2002, 2005). Such behavioral changes may alter herbivore feeding patterns, changing patterns and intensity of plant tissue damage and the exposure of the plant to oral secretions of herbivores. Plant volatile emissions are likely to be affected by such changes (Schmelz et al., 2003; Pare et al., 2005).

Whereas qualitative and quantitative changes in volatile emissions have been shown for undamaged Bt cotton and mechanically damaged GM tomatoes (Smith et al., 1996; Yan et al., 2004), the effects of genetic engineering on herbivore-induced plant volatiles remain largely unexplored. No change in parasitoid attraction was

found between Bt and non-Bt oilseed rape plants equally damaged by Bt-resistant herbivores, suggesting a similarity in the volatiles produced by each plant, but no mechanism was elucidated (Schuler et al., 1999b). Further understanding of these effects will be accomplished most readily in plant species, such as maize, where the molecular and biochemical bases of induced volatile production are well understood. In response to herbivore feeding on maize, specific blends of compounds from the lipoxygenase, terpenoid biosynthesis, and shikimic acid pathways are released both locally and systemically (Turlings and Tumlinson, 1992). Whereas many of these compounds are not released in response to mechanical damage, the application of herbivore regurgitant induces a volatile response that closely resembles actual feeding (Turlings et al., 1990).

In this study, we investigated how induced volatiles varied between GM maize with the Bt *cryIA(b)* gene inserted and a nontransformed inoline. The endotoxin produced by the modified gene is targeted toward the European corn borer (*Ostrinia nubilalis* Hübner), but is also effective at increasing developmental times and mortality rates in other lepidopteran larvae, including the corn earworm (*Helicoverpa zea* Boddie) (Wiseman et al., 1999). *H. zea* is an economically important pest of maize kernels, but can also cause damage to whorl-stage maize plants by foliar feeding (Capinera, 2000). We first compared volatiles from undamaged plants to determine if Bt and non-Bt maize plants differed in their constitutive release of chemicals. Then, we performed several experiments comparing volatile induction by *H. zea* feeding on Bt and non-Bt maize plants, manipulating larvae numbers, damage amounts, and feeding patterns. To further control damage levels and patterns, we mimicked herbivore feeding by mechanically wounding leaves and applying regurgitant collected from larvae fed either with Bt or non-Bt maize.

Methods and Materials

Plants and Insects

Both Bt and non-Bt maize seeds were obtained from Dekalb (Monsanto Co., St. Louis, MO, USA). The Bt maize DKC61-25 contains the Monsanto Event 810, with the *cryIA(b)* gene. The nearest inoline of the Bt hybrid, DKC61-24, was used for the non-Bt plants. Plants were grown in a growth chamber (14-hr photophase; 25:22°C day/night, 65% RH) in pots (16 × 17-cm diam) filled with a peat-based general-purpose potting soil with micronutrients added.

H. zea larvae were reared from eggs (USDA/ARS, Tifton, GA, USA) on an artificial casein-based diet in plastic diet cups (30 ml, Solo Cup Co., Urbana, IL, USA) in incubators (14-hr photophase, 25°C, 60% RH). Larvae were starved for 24 hr before experiments. Third instar larvae were placed on plants for all experiments.

Volatile Collection and Analysis

We collected volatiles in a greenhouse from potted, intact 2- to 3-wk-old maize plants using a closed push/pull system (Analytical Research Systems, Gainesville, FL, USA). About 3 in. above the soil line, a Teflon® base consisting of two sliding

plates (that when pushed together left a hole for the stalk to pass) was secured around the plant. A cotton ball was wrapped around the stalk to fill space between the base and plant and to allow air to exit. A cylindrical glass chamber (46 cm tall, 8-cm diam) was placed over the plant, resting on the Teflon[®] base. Air was pumped into the chamber (3.0 l/min) through Teflon[®] tubing and pulled out of the chamber (1.0 l/min) through side ports and across traps containing 40 mg SuperQ[®] (Alltech, Deerfield, IL, USA). Two separate volatile collections were made daily: one during the light phase for 12 hr, and the other during the dark phase for 8 hr.

Traps were eluted with 150 μ l of dichloromethane, and *n*-octane (80 ng) and nonyl acetate (400 ng) were added to each sample as internal standards. Volatiles were analyzed by gas chromatography with a Hewlett-Packard model 5890 GC. Samples were injected in 1- μ l aliquots with a splitless injector held at 240°C. The column (15 m \times 0.25 mm i.d., HP-1) was maintained at 60°C for 2 min, and then increased 4°C/min to 180°C, where it was held for 10 min. Quantifications of compounds were made relative to the internal standard using Enhanced Chem-Station software (Agilent Technologies, Palo Alto, CA, USA). Identifications were confirmed with mass spectrometry and by comparing retention times with those of known standards.

Undamaged Plants

Volatiles were collected from undamaged Bt and non-Bt maize to determine baseline emissions. Collections were made from four plants of each type. Means for Bt and non-Bt maize for this and all other experiments were compared with Student's *t* test using Minitab v. 14.1 (State College, PA, USA) (Sokal and Rohlf, 1995).

Equal Number of Larvae Per Plant

We tested the induced responses of maize when exposed to an equal number of larvae on each plant type to approximate likely field conditions. Two third instars were added to each chamber containing individual Bt or non-Bt plants and allowed to feed freely on plants during 4 d of volatile collecting. Each day, dead or molting larvae were replaced with ones that had been starved for 24 hr. These comparisons were made on six Bt and six non-Bt plants.

To avoid disturbing the plants while larvae were feeding, we assessed herbivore damage while plants remained inside volatile collection chambers. Each morning during the 4-d feeding experiment, we estimated the size of damage holes on unfurled leaf blades and through the enumerated layers of the whorled cylinder by comparison with a guide composed of five different-sized circles of known area. From the number and estimated size of holes, we calculated the total leaf area removed.

Equal Amount of Herbivore Damage

To determine if the amount of larval feeding affects induced responses of Bt and non-Bt maize, we added *H. zea* larvae to Bt plants daily to achieve equivalent damage on both plant types. On the first day of the experiment, 8–10 third instars were added to each Bt maize plant, and one or two larvae were added to each non-Bt plant. Each day, more third instars (~5) were added to Bt maize through the tops

of chambers to replace ones that had died. By the end of the 4-d sampling period, most larvae had reached their fourth instar. As a control, the tops of the non-Bt chambers were vented for an equal time. Each day, we assessed and estimated the amount of plant damage as above. Three plants of each type were used for comparisons.

Equal Pattern and Amount of Herbivore Damage

Individual larvae on non-Bt plants feed longer and cause greater localized damage. Because the feeding pattern (i.e., the number and size of damage holes) may influence induced responses, we manipulated the duration of larval feeding, resulting in the consumption of an equal amount of leaf tissue in a similar pattern on each plant type. Third instars placed on Bt and non-Bt maize plants were monitored continuously as they fed, and individual larvae were removed once they consumed an area of leaf tissue equivalent in size to the damage a Bt plant would typically incur from one larva ($\sim 5 \text{ mm}^2$). The number of larvae on each plant was manipulated to achieve similar amounts of damage across plants ($\sim 60 \text{ mm}^2$). After all larvae were removed, usually about 3 hr after the first larvae were placed on the plants, we collected volatiles in a growth chamber for the next 3 d by using a collection system similar to the one used in the other experiments (Analytical Research Systems). Air was pumped into the chambers at 2 l/min and pulled out through traps at 0.8 l/min. A total of five Bt plants and four non-Bt plants were used as replications in this experiment. When volatile collections were finished, we cut plants at the base and digitally scanned leaves to quantify leaf area removed (SigmaScan Pro v. 5.0, SPSS Inc., Chicago, IL, USA).

Mechanical Damage and Regurgitant Application

To control the pattern and amount of damage more precisely than was possible using natural herbivory, we artificially simulated herbivory by mechanically damaging leaves and applying larval regurgitant. We collected regurgitant from fourth instar *H. zea* by probing the preoral cavity with a pipette tip and drawing in the fluid released from the mouth. Larvae in diet cups had either been fed with foliage from non-Bt maize for 24 hr or foliage from Bt maize for 6 hr after having been starved for 18 hr prior to collecting. The shorter feeding time was necessary for those fed with Bt maize because the larvae would become inactive soon after eating the transgenic leaves, at which point regurgitant could not be obtained. Regurgitant was kept on ice while being collected and boiled for 3 min to stop enzyme activity (Mori et al., 2001). Samples were stored at -80°C until needed. Preliminary experiments showed no clear differences between the volatile profiles of Bt or non-Bt plants induced by regurgitant collected from larvae fed with either Bt leaves or non-Bt leaves (data not shown). Because of this similarity in responses and the difficulties involved in collecting regurgitant from larvae exposed to the Bt toxin, regurgitant collected from non-Bt fed larvae was used to compare volatiles from Bt and non-Bt plants with artificial herbivory.

Volatiles were collected from mechanically damaged plants with and without regurgitant. Plants were damaged by scraping leaf tissue from the upper side of

leaves with a razor blade in a 1 × 2 cm rectangle. Each day, the glass volatile collection chambers were temporarily removed, and three leaves on each plant were damaged, with regurgitant (10 µl) applied to each damaged spot. The chambers were replaced, and volatiles were collected. Three Bt and three non-Bt plants were used for each experiment (with and without regurgitant).

Results

Undamaged Plants

We found no differences in the total amount of volatiles released or in quantities of individual compounds in the emissions from undamaged Bt and non-Bt maize (Table 1).

Equal Numbers of Larvae Per Plant

When equal numbers of *H. zea* larvae were fed on both maize isolines, non-Bt plants suffered 4 to 22 times more damage by the end of the experiment (Student's *t* test, $t_6 = -3.53$, $P = 0.012$). By the third day, the amount of damage as well as the amount of volatiles released was significantly greater on non-Bt plants (Table 1). Damage to Bt maize typically occurred only on the first day larvae that were placed on the plants, because after initial feeding, larvae often would be inactive but still alive for one or two more days. On non-Bt plants, larvae would continue to feed for the 4 d of the experiment, causing extensive damage.

Equal Amount of Herbivore Damage

When we equalized the amount of herbivory each day, there was a significant difference in the total amount of volatiles released by day 3 (Table 1). Four

Table 1 Total volatiles (Mean ± SE) released during and damage incurred (mm²) preceding day 3 from Bt and non-Bt maize

Treatment	Type	Total volatiles (ng/hr)	<i>P</i> value	Total damage (mm ²)	<i>P</i> value
Undamaged control	Bt	180.7 ± 41.6	0.834	0	n/a
	Non-Bt	193.5 ± 41.7		0	
Equal number of larvae per plant	Bt	652.7 ± 236.7	0.003	39.4 ± 23.3	0.006
	Non-Bt	3118.6 ± 482.9		302.4 ± 59.8	
Equal amount of herbivore damage	Bt	3869.3 ± 413.5	0.049	159.7 ± 25.3	0.519
	Non-Bt	2275.7 ± 274.8		139.3 ± 6.8	
Equal pattern and amount of herbivore damage	Bt	1192.0 ± 65.3	0.419	62.1 ± 6.2	0.913
	Non-Bt	1300.0 ± 115.5		61.0 ± 7.3	
Mechanical damage	Bt	949.6 ± 293.1	0.959	600.0	n/a
	Non-Bt	925.0 ± 330.5		600.0	
Mechanical damage + regurgitant application	Bt	1521.2 ± 460.1	0.908	600.0	n/a
	Non-Bt	1614.7 ± 587.2		600.0	

Bolded numbers signify statistically significant differences between Bt and non-Bt values (Student's *t* test, $P < 0.05$).

Table 2 Major compounds released from Bt and non-Bt maize receiving an equal amount of herbivore damage on day 3^a

Compound	Bt	Non-Bt	<i>P</i> value
(<i>E</i>)-β-Farnescene	867.4 ± 148	632.6 ± 150	0.347
Bergamotene	177.6 ± 31	124.8 ± 25	0.275
(<i>E</i>)-β-Caryophyllene	23.3 ± 2	16.6 ± 5	0.308
Geranyl acetate	205.2 ± 31	75.9 ± 17	0.034
Indole	257.6 ± 20	293.4 ± 154	0.839
Linalool	1000.9 ± 89	443.4 ± 94	0.023
β-Myrcene	46.6 ± 5	22.58 ± 4	0.035
(<i>E</i>)-4-8-Dimethyl-1,3,7-nonatriene	606.7 ± 112	318.6 ± 89	0.138
(<i>E,E</i>)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	151.3 ± 27	82.2 ± 19	0.131
Unknown 1	23.1 ± 9	5.5 ± 1	0.182
Unknown 2	57.1 ± 2	40.5 ± 22	0.530
Unknown 3	45.0 ± 8	29.7 ± 7	0.244
Unknown 4	35.4 ± 6	23.2 ± 5	0.231
(<i>Z</i>)-3-Hexenyl acetate	83.7 ± 19	21.6 ± 10	0.066

^a Values represent means ± SE in ng/hr.

Bolded numbers signify statistically significant differences between Bt and non-Bt values (Student’s *t* test, *P* < 0.05).

compounds constituted a major proportion of this difference: (*Z*)-3-hexenyl acetate, linalool, β-myrcene, and geranyl acetate (Table 2). The pattern of damage inflicted by each larvae differed between plant lines, however, with larvae on Bt plants feeding in short bouts and leaving many small, scattered holes, whereas larvae on non-Bt plants fed more or less continuously, creating large areas devoid of leaf tissue (Fig. 1).



Fig. 1 Sample of feeding patterns created by *Helicoverpa zea* larvae on Bt and non-Bt maize leaves

Table 3 Characteristics of damage holes created by *Helicoverpa zea* larvae while controlling duration of feeding to achieve an equal amount and pattern of damage

	Bt	Non-Bt	<i>P</i> value
Number of holes	15.5 ± 3.6	17.0 ± 3.7	0.789
Area (mm ²)	62.1 ± 6.2	61.0 ± 7.3	0.913
Perimeter (mm)	122.6 ± 11.0	126.9 ± 9.4	0.793

Equal Pattern and Amount of Herbivore Damage

Manipulating the amount of time larvae were allowed to feed resulted in plants with similar numbers of damage holes, leaf area removed, and perimeters of damaged areas (Table 3). In this case, the two maize isolines emitted similar blends in terms of total volatiles released and proportions of individual compounds (Table 1).

Mechanical Damage and Regurgitant Application

The volatiles released by Bt and non-Bt plants after applying larval regurgitant did not differ substantially from one another quantitatively or qualitatively (Table 1).

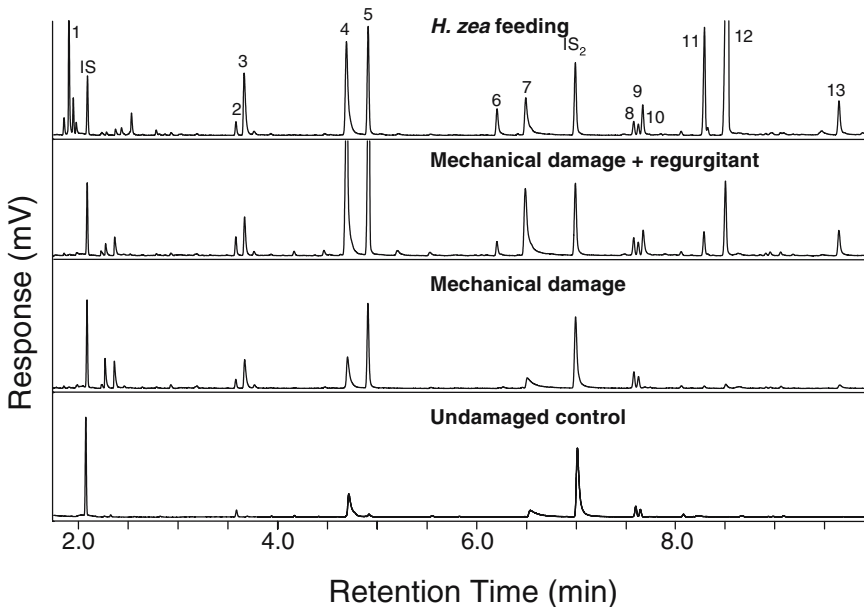


Fig. 2 Chromatograms of volatile compounds emitted on day 3 from non-Bt maize plants subjected to feeding by *H. zea* larvae, mechanical damage, mechanical damage with regurgitant applied, and no damage. Major compounds (with a threshold emission of 2 ng/hr) are labeled as follows: (1) unknown 1; (2) β -myrcene; (3) (*Z*)-3-hexenyl acetate; (4) linalool; (5) (*E*)-4-8-dimethyl-1,3,7-nonatriene; (6) unknown 2; (7) indole; (8) unknown 3; (9) unknown 4; (10) geranyl acetate; (11) bergamotene; (12) (*E*)- β -farnescene; (13) (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; IS and IS₂, internal standards (*n*-octane and nonyl acetate)

The volatile profile of maize plants with artificial herbivory closely approximated that of plants exposed to actual larvae damage (Fig. 2). However, regurgitant-treated plants did not release some “green leafy volatiles” (Turlings et al., 1998) and had a weaker induction of β -farnescene and bergamotene (Fig. 2). Plants that were wounded with a razor blade appeared to release fewer volatile compounds and smaller quantities of the ones that were emitted relative to those damaged by herbivores.

Discussion

In our experiments, genetic modification did not appear to alter the volatile profile of undamaged maize (Table 1). However, undamaged Bt cotton plants emitted unique compounds and different proportions of typical compounds when compared to non-Bt cotton (Yan et al., 2004), hinting at the diversity of effects genetic modification can have on different plant species.

Undamaged Bt maize plants do not appear to influence the ovipositional preferences of some Lepidopteran pests (Hellmich et al., 1999; Liu et al., 2002), suggesting that an equal number of larvae would initially be expected on each plant type. When we allowed equal numbers of larvae to feed on plants continuously for 4 d, non-Bt maize received up to 22 times more damage and emitted significantly more volatiles than Bt maize (Table 1). In the field, similar reductions in induced volatile emissions could cause areas planted with Bt maize to be less attractive to natural enemies that rely on herbivore-induced plant cues to find suitable hosts. Sked (2003) showed that fields sown with Bt maize had one third the numbers of *Macrocentrus cingulum*, a parasitoid specialist on the European corn borer, as non-Bt plots. Such reductions in population size could conceivably lead to local extirpation of natural enemies. At the same time, reduced volatile emissions could attract herbivores seeking a suitable oviposition site. For example, *O. nubilalis* oviposits in the field preferentially on undamaged maize plants compared to those infested with conspecific larvae (Harmon et al., 2003). More pest egg masses laid on Bt plants emitting fewer volatiles than severely damaged non-Bt maize could increase the possibility of resistance emerging within pest populations (Hellmich et al., 1999).

If an ovipositional preference for Bt plants did arise in the field, the greater numbers of larvae might produce an amount of damage closer to that incurred by non-Bt plants. Our results indicate that even with an equal amount of damage, volatile emissions still varied between Bt and non-Bt plants (Table 2). Linalool, β -myrcene, and geranyl acetate were released in significantly greater quantities from Bt maize. These compounds have been implicated in natural enemy attraction through behavioral studies and antennal electrophysiological responses (Turlings et al., 1991; Rose et al., 1998; Gouinguene et al., 2005).

Although damage amounts were equalized by increasing the number of larvae on Bt plants, these larvae exhibited a different feeding behavior, and thus created a different pattern of damage than larvae feeding on non-Bt plants. Damage holes were more numerous but smaller on Bt maize, increasing the perimeter of damaged areas and maximizing the contact zone of oral secretions and exposed plant tissue. When the feeding of individual larvae was manipulated to achieve similar damage

patterns, differences in volatile emissions between Bt and non-Bt maize were no longer evident, suggesting that these differences resulted from the altered feeding behavior of larvae on Bt plants, rather than physiological changes in plant response resulting from genetic modification.

When the damage pattern and amount were standardized, either by manipulating the feeding duration of larvae or by applying larval regurgitant to mechanically damaged leaves, the induced volatile emissions of Bt and non-Bt maize were similar. These results suggest that Bt and non-Bt fields subjected to similar amounts and patterns of feeding damage, as might occur if pest populations developed resistance to the Bt maize, would be equally attractive to natural enemies. Similarly, the parasitic wasp *Cotesia plutellae* was found to be equally attracted to Bt oilseed rape plants fed upon by Bt-resistant hosts (*Plutella xylostella*) and wild-type plants equally damaged by the same herbivore, suggesting no change in the composition of the induced volatiles (Schuler et al., 1999b).

In summary, observed differences in induced volatile profiles between Bt and non-Bt maize appear to result from different amounts and patterns of feeding damage inflicted. Individual larvae on transgenic plants fed in short bouts, causing less damage and leaving distinctive damage patterns relative to conventional plants. When we controlled for these differences in the amount and pattern of feeding damage inflicted, differences in the volatile profiles of Bt and non-Bt plants disappeared, suggesting that these differences are a consequence of altered larvae feeding behavior on Bt plants rather than of changes in biochemical plant defense pathways. As reduced herbivory is the goal of insect-resistant transgenic crops, the consequent reductions in volatile emissions may have important implications for sustainable pest management.

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