Mississippi State University

Scholars Junction

12-10-2010

Detection And Characterization Of A Volatile Compound As A Response To Fall Armyworm (Spodoptera Frugiperda) Feeding In Maize (Zea Mays)

Whitney Elizabeth Crow Smith

Follow this and additional works at: https://scholarsjunction.msstate.edu/td

DETECTION AND CHARACTERIZATION OF A VOLATILE COMPOUND AS A

RESPONSE TO FALL ARMYWORM (*SPODOPTERA FRUGIPERDA*)

FEEDING IN MAIZE (*ZEA MAYS*)

By

Whitney Elizabeth Crow Smith

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Masters of Science
in Agricultural Life Science (Biochemistry)
in the Department of Biochemistry and Molecular Biology

Mississippi State, Mississippi

December 2010

DETECTION AND CHARACTERIZATION OF A VOLATILE COMPOUND AS A

RESPONSE TO FALL ARMYWORM (*SPODOPTERA FRUGIPERDA*)

FEEDING IN MAIZE (*ZEA MAYS*)

By

Whitney Elizabeth Crow Smith

Approved:

_____
Ashli Brown
Assistant Professor of Biochemistry and
Molecular Biology
Major Professor

_____
Scott Willard
Professor and Head of Biochemistry
and Molecular Biology
Committee Member

_____
W. Paul Williams
Adjunct Professor of Biochemistry and
Molecular Biology
Committee Member

_____
Din-Pow Ma
Professor of Biochemistry and
Molecular Biology
Graduate Coordinator

_____
George Hopper
Interim Dean of the College of
Agriculture and Life Sciences

Name: Whitney Elizabeth Crow Smith

Date of Degree: December 10, 2010

Institution: Mississippi State University

Major Field: Agricultural Life Science (Biochemistry)

Major Professor: Dr. Ashli Brown

Title of Study: DETECTION AND CHARACTERIZATION OF A VOLATILE COMPOUND AS A RESPONSE TO FALL ARMYWORM (*SPODOPTERA FRUGIPERDA*) FEEDING IN MAIZE (*ZEA MAYS*)

Pages in Study: 62

Candidate for Degree of Masters of Science

Maize (*Zea mays)* is an important agricultural crop frequently targeted by pests that pose a threat to plant development and survival. To deal with this problem, maize generates a wide variety of responses to attack by pests, from activation of wound-response pathways to the release of volatile compounds. Several maize lines have been developed that show resistance to one common pest, the larvae of the fall armyworm (*Spodoptera frugiperda*). Analysis of the volatiles released by the resistant and susceptible lines in the presence and absence of the fall armyworm was conducted using SPME coupled to GC/MS. Caryophyllene, a commonly released plant volatile, was identified in the resistant line. In the susceptible line, caryophyllene was detected in smaller quantities or not at all. The results of a preference study demonstrated that fall armyworm larvae show a statistically significant preference for yellow-green whorl tissue from the susceptible over the resistant line.

ACKNOWLEDGEMENTS

There are many people I would like to thank who were instrumental in the preparation of this thesis. I would like to express my sincere gratitude to Dr. Ashli Brown for taking me on as her graduate student and for her help and guidance. To Mr. Bill Holmes and Mrs. Beth Thomas, thank you for allowing me to intrude into your lab and teaching me to use all the equipment that was required for this research. Thanks to Dr. Paul Williams for all his assistance, and to Dr. Willard for his support.

To Erik Mylorie, thank you so much for all your advice on laboratory procedures and help with statistical analysis. Thanks to Erik, Candace Williams, Ashley Meredith, Katherine McGinley, and Chandler Pace for your friendship and making our office a fun place to work.

A special thanks to Chandler, Brett Collins, and Will Ford for your help with greenhouse work and in the laboratory. Each of you was always willing to help me with anything I needed, and I appreciate that greatly.

To Renuka Shivaji, I give heartfelt thanks for all your assistance. You taught me almost everything I know how to do in the laboratory, and you were always available to help me troubleshoot or teach me new techniques any time I needed you.

Finally, to my family: I could have never done this without your constant encouragement and support. Mom, Dad, and Mallory, I love you all so much, and thank you for everything you have done for me. To my wonderful husband, Chad, I cannot put into words my appreciation to you for your thesis-writing advice, help finding resources,

and your encouragement during the times I thought I would never be able to do this. You were always there when I needed you, and I thank you from the bottom of my heart for your love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

Maize

Maize (*Zea mays*) is an important agricultural crop grown throughout the Midwestern and Southeastern United States. In 2008, farmers in the United States planted 86 million acres of maize, yielding 12.1 billion bushels at an average price of $3.90 per bushel. The 2008 maize crop was valued at $47.19 billion, more than the value of oats, barley, sorghum, wheat, and soybeans (World of Corn Report 2009). As with any commercially produced crop, controlling pests in the field has always been an important part of the growing process. Maize is susceptible to a wide variety of pests, including herbivorous larvae in the order Lepidoptera such as the fall armyworm (FAW, *Spodoptera frugiperda),* corn earworm (*Helicoverpa zea),* and the southwestern corn borer (*Diatraea grandiosella).* Annual economic loss from maize damaged by fall armyworm larvae can range from 300 to 500 million dollars (Fall Armyworm Agronomic Spotlight 2010). There are also a variety of pests that feed on the maize root, such as the western corn rootworm (*Diabrotica virgifera)* and the corn root aphid (*Anuraphis maidiradicis)* (Corn Insect Pests 1998). The black cutworm (*Agrotis ipsilon)* cuts off a maize seedling and moves the seedling to its burrow, where it eats it (Catchot 2010). In addition, there are several piercing and sucking insects such as the corn leaf aphid (*Rhopalosiphum maidis)* and the spider mite (*Tetranychus* sp.) that cause extensive damage to the young maize plants (Corn Insect Pests 1998).

1

Insecticide Use in Maize

Insecticides have been used to control pests in maize nearly as long as maize has been commercially cultivated. The first insecticides were often inorganic compounds, such as sulfur, and insecticidal soaps. Boric acid, which is fatal to insects only after it has been eaten, was also an early insecticide and is still used against several household pests. Modern insecticides fall into three main categories according to their active ingredient: pyrethroids, carbamates, and organophosphates (Pedigo 2002).

The first pyrethroid, allethrin, was developed in the 1940s. Currently, fourth-generation pyrethroids, such as cyfluthrin, prallenthrin, and flucythrinate, are often used to control insect pests. These synthetic compounds, which are derivatives of the naturally found pyrethrins from the flowers of *Chrysanthemum* species, work by binding to the voltage-gated sodium channel and impeding its inactivation (Hopkins and Pietrantonio 2009). Pyrethroids are not toxic to mammals at low doses, and are broken down naturally by ultraviolet light from 4 to 7 days post application (Pedigo 2002).

Carbamates were first produced from carbamic acid in the 1950s. The two most common carbamates are carbaryl (for example, Sevin®), which is commonly used to kill insects in fruit production, and carbofuran, a soil-applied insecticide that is effective against nematodes and corn rootworm (Pedigo 2002). Carbamates reversibly inhibit acetylcholinesterase, an enzyme that degrades acetylcholine. Acetylcholine builds up in the synaptic cleft of the insects, neurons continue to transmit their electrical charge, and death occurs from the overstimulation of the nervous system (Brown 2006).

The insecticidal properties of organophosphates were discovered in Germany during World War II because of their relationship to the "nerve gases." These compounds work similarly to the carbamates, but inhibit acetylcholine irreversibly by

phosphorylating it (Kitz and Wilson 1962). Organophosphates are derived from phosphoric acid, are unstable in the presence of light, and are some of the most toxic and widely used insecticides (Pedigo 2002).

While insecticides are extremely effective at controlling pests in maize and other commercially important crops, their environmental and health risks are extensive. Insecticides are rigorously tested and regulated by the EPA, and insecticide applicators must operate with extreme caution. The EPA regulates every facet of insecticide use and application, from the amount that can be sprayed, how often the insecticide can be applied, how close to open bodies of water the insecticide can be sprayed and much more. Users of restricted-use pesticides must be certified by the EPA, and applicators are required to keep accurate spray records.

The mode of action of most insecticides, specifically organophosphates and carbamates, are not specific to insects, but will harm humans and other animals, as well. The LD_{50}, or the number of milligrams of pesticide per kilogram of body weight that will kill 50% of the population, is as low as a few drops for some of the most toxic insecticides. For example, the oral LD_{50} of disulfoton, an organophosphate, is 2.3 mg per kg of body weight (approximately 50 ppm), and the dermal LD_{50} is 6 mg/kg. Insecticides can harm the environment, as well, killing "good" insects and causing harm to other animals if they accidentally ingest it. A list of several common insecticides and their properties are given in Table 1.1.

Table 1.1   Common maize insecticides and their properties.

Common Name	Trade Name	Class	LD_{50}, Oral	LD_{50}, Dermal	Insects Controlled (Maize)
Biphenthrin	Capture®, Talstar®	Pyrethroid	375	>2000	Rootworms, wireworms, cutworms, armyworms, corn borer, leaf aphids
Carbaryl	Sevin®, Sevimol®	Carbamate	850	>4000	Cutworms, armyworms, corn borer
Carbofuran	Furadan®	Carbamate	8	>10,200	Rootworm, corn borer
Chlorpyrifos	Dursban®, Lorsban®	Organophosphate	125	385	Rootworm, wireworms,cutworms, armyworms, corn borer, leaf aphids
Chlorethoxyfos	Fortress®	Organophosphate	1.8	12.5	Wireworms, rootworms
Lambda cyhalothrin	Warrior®, Karate®	Pyrethroid	56	623	Cutworms, armyworms, corn borer, corn flea beetles

The cost of insecticides is another concern. The cost of insecticides is often $2-20 per acre, causing the price of the maize, cotton, or other crop to rise and leaving farmers with less profit. If the pests are not detected and insecticide applied early enough, widespread loss of the crop can occur. Therefore, the management of pests by natural and biological means, as well as ecological management, is becoming the preferred method of insect control.

Biological Control

The control of agronomic pests by biological methods has increased in popularity over the last several decades. Biological control was first established in the United States in 1888 when the vedalia beetle, *Rodolia cardinalis,* was introduced to eliminate the cottony cushion scale, *Icerya purchasi,* in citrus (Pedigo 2002). Recently, fast, reliable

DNA sequencing has allowed the genome of maize to be sequenced, and the insertion of resistance genes into commercial hybrids is now commonplace. Hybrids engineered to be resistant to the common herbicide glyphosate (Roundup®) are now the norm. Insect resistance traits have also been conferred to maize lines through the discovery of the insecticidal properties of *Bacillus thuringiensis*.

B. thuringiensis (Bt) is a bacterium that naturally occurs in the soil. It produces spores coated in endotoxin proteins, which differ in their specificity (Chungjatupornchai et al. 1988). Toxins have been identified that are specific to Lepidoptera (Hofte et al. 1986), Diptera (Chungjatupornchai et al. 1988), and many other insect larvae. These protoxins, about 130-140 kDa, are processed in the midgut of the insects into a 60 kDa active toxin (Van Rie et al. 1989). Different strains of *B. thuringiensis* produce different toxin proteins, and research suggests differences between the proteins are a result of homologous recombination during evolution (Hofte et al. 1986). These different toxins bind to specific binding sites on the membranes of the insects' midgut epithelial cells, and there are also differences in the concentration of the binding sites in different insects (Van Rie et al. 1989).

Several commercial maize lines are available that have been engineered with various *B. thuringiensis* toxin genes, called Cry genes. Several of the traits are listed in Table 1.2. Manufacturers often "stack" *Bt* traits with other traits, such as resistance to common herbicides. While these varieties provide excellent control of corn earworm and corn borer, they do not provide adequate control of the larvae of *Spodoptera frugiperda,* the fall armyworm (Farrar et al. 2009, Chilcutt et al. 2007). Additional methods of control, such as the application of insecticides, are often needed during years with extreme fall armyworm infestations. Thus, the development of a line of maize resistant

to the fall armyworm is of upmost importance to the agricultural community. Any trait or traits identified as conferring resistance to the fall armyworm larvae could be engineered into current lines of *Bt* maize to provide resistance to more common maize pests.

Table 1.2   Several *Bt* traits and their manufacturers.

Trait Name	Manufacturer
YieldGard VT Double/Triple Pro®	Monsanto
Genuity SmartStax®	Monsanto
Agrisure® CB/LL	Syngetna
Herculex®	Dow Agrosciences/Pioneer Hi-Bred

Fall Armyworm

The larvae of the fall armyworm (Figure 1.1, *Spodoptera frugiperda)* are considered a persistent pest of young maize (*Zea mays)* plants. Most members of the order Lepidoptera, which includes the fall armyworm, have a life cycle that includes both a larval, caterpillar stage, as well as an adult moth or butterfly stage. The fall armyworm does not have the ability to go dormant and thus spends winters in South Florida and Texas, then disperses throughout the Central and Eastern United States and as far north as southern Canada (Sparks 1979). Adult moths reach Mississippi between April and June and lay egg masses, often on the leaves of young corn plants; the larvae hatch and begin feeding on the surrounding tissue. The full-grown larvae of the fall armyworm build cocoons in the soil using silk produced by modified salivary glands; they emerge from these cocoons as an adult moth (Pedigo 2002, Sparks 1979). The larvae of this order feed voraciously on plants; almost every plant species has at least one caterpillar that feeds on

it (Pedigo 2002). Severe fall armyworm outbreaks occur sporadically throughout the Central and Eastern United States; a severe outbreak in 1977 caused losses of $137.5 million in Georgia alone (Sparks 1979). Outbreaks are more common when conditions are favorable in the overwintering sites; cool, wet springs followed by warmer, humid weather and heavy rainfall often produce large numbers of fall armyworms (Sparks 1979).

Figure 1.1   The larvae of the fall armyworm, *Spodoptera frugiperda.*

Resistance of Maize to Fall Armyworm Larvae

A line of maize, Mp708, which shows a degree of resistance to feeding by fall armyworm larvae, has been developed through traditional plant breeding methods (Figure 1.2, Williams et al. 1990). Mp708 was developed by crossing Mp704 and Tx601, then selfing resulting selections for eight generations (Williams et al. 1990). Mp704 displays some resistance to fall armyworm, but Mp708 was shown to have better pollen and seed production (Williams et al. 1990). Mp704 has tropical origin; it was developed by

crossing Mp496 with an S2 from Republica Dominica Gpo. 1 (Williams and Davis 1982). Mp707 is another resistant line developed from Caribbean exotic germplasm (Davis et al. 1999). Tx601 is an inbred line of maize developed in Texas that is susceptible to the fall armyworm (USDA-ARS National Genetic Resources Program).
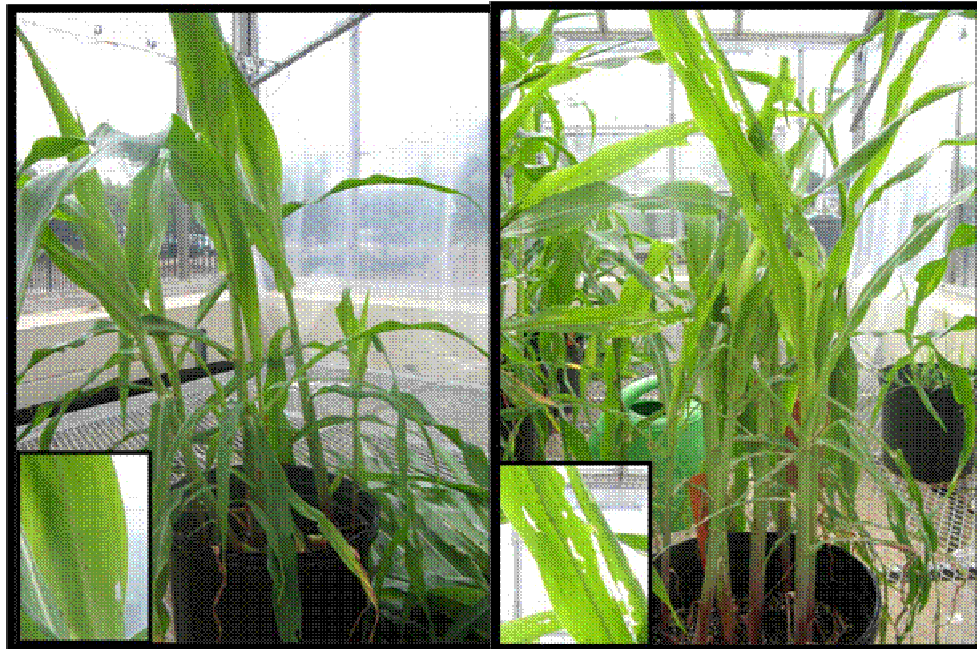
Figure 1.2   Mp708 (left) and Tx601 (right) after infestation by fall armyworms. Tx601, a susceptible line, has extensive leaf feeding damage, while Mp708 has very little.

Extensive research has demonstrated that fall armyworm larvae reared on diets of either fresh or reconstituted yellow-green whorl tissue from Mp707 crossed with Mp708 (both resistant lines) were significantly smaller than larvae fed a similar diet from a line susceptible to feeding by fall armyworm (Davis et al. 1999). Larvae reared on a diet of Mp708 yellow-green whorl tissue also had a longer developmental period, lower growth rate, and lower efficiency of conversion of ingested and digested food to body substance than larvae fed a diet of only susceptible maize tissue (Chang et al. 2000). Similarly, fall

armyworm larvae fed diets of callus developed from resistant embryos weighed significantly less after seven days than those fed callus from susceptible maize. In addition, when fall armyworm larvae were given a choice between resistant or susceptible callus, two times as many larvae preferred the susceptible callus (Williams et al. 1985).

The method of resistance of Mp708 is not fully understood. The cell wall complex and cuticle are 1.7x thicker in the resistant line than a susceptible line, and the inner whorl tissue of the resistant line was tougher than tissue from the susceptible line (Davis et al. 1995). Resistant hybrids transitioned from juvenile to adult stage earlier than susceptible hybrids, and resistant hybrids had a higher level of hemicellulose (Williams et al. 1998, Williams et al. 1999). Mp708 has been shown to have a moderately high constitutive expression of jasmonic acid (JA) and other octadecanoid compounds prior to infestation by fall armyworm. On the other hand Tx601, a genotype susceptible to feeding by fall armyworm, activates the JA pathway only in response to feeding, suggesting that Mp708 is "primed" to respond swiftly to an attack (Shivaji et al. 2010).

Increased defense proteins in Mp708 as compared to a susceptible line upon attack by the fall armyworm larvae also plays a role in resistance (Chen et al 2009). In addition, the presence of Mir1-CP, a unique defense protein, in the yellow-green whorl region of a resistant—but not susceptible—hybrids after infestation with fall armyworms has been noted (Pechan et al. 2000). The protein was determined to damage the peritrophic matrix, which separates the food from the midgut, of the fall armyworm larvae (Pechan et al. 2002). Clearly, Mp708 has a specific plant defense mechanism that confers resistance to fall armyworm larvae.

Plant Defense Mechanisms

Plants have highly sophisticated defense mechanisms against various biotic and abiotic stresses. Without the option to evade pests by mechanical means such as simply running away, plants have evolved multiple complex methods of defense, both through biochemical signaling pathways and physiological changes. However, implementing these defenses requires a large energy input from the plant, and thus the prevention of attacks by pests is also equally important. There are two different types of defenses: those expressed constitutively and those that are induced by the presence of pests or environmental conditions. One such pathway is the octadecanoid pathway, which produces jasmonic acid and is induced by the infestation of the plant by chewing and tearing herbivores, such as the fall armyworm (*Spodoptera frugiperda)* and other members of the order Lepidoptera (Shivaji et al. 2010).

A plant has innumerable defense pathways, including those activated in response to pathogens that directly attack the plant. Insects that pierce or suck nutrients from the phloem, as well as bacteria, fungal, or viral pathogens, all elicit a similar response from the plant (Walling 2000). In addition to activation of signaling response pathways such as the octadecanoid, salicylic acid, and isoprenoid pathways, attack by a pathogen leads to the production of reactive oxygen species and nitric oxide (Walling 2000). There are three main classes of molecules involved in signaling pathways: salicylic acid and its methyl conjugate, methyl salicylate; jasmonic acid; and ethylene (Rojo et al. 2003). In addition, abscisic acid (ABA), a plant hormone that plays a role in plant growth, development, and response to stress, has recently been shown to also be involved in plant defense mechanisms (Maksimov 2009). Targeted protein degradation by the ubiquitin pathway also plays an important role in the ethylene and jasmonate signaling pathways

by degrading transcription factors and other proteins that play a role in their synthesis. (Dreher and Callis 2007).

Salicylic Acid

Salicylic acid (Figure 1.3, SA) is synthesized by two different pathways in plants; it can be synthesized from chorismate or phenylalanine. Upon a pathogen attack, SA production is upregulated; the signaling pathway is controlled by at least two mechanisms, one which requires the *NPR1* gene and one that does not. *NPR1* expression is increased when a plant is attacked by a pathogen and SA accumulates; SA also stimulates NPR1 to move into the nucleus and interact with DNA binding proteins that lead to the expression of pathogenesis-related (PR) proteins. Alternately, research suggests a *NPR1*-independent method for expression of PR proteins is also present, but this pathway is not well understood (Loake and Grant 2007, Shah 2003). The accumulation of SA is required for the induction of systemic acquired resistance (SAR) (Devoto et al. 2003).
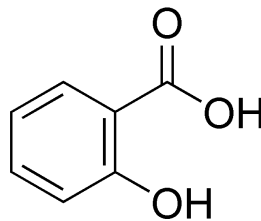
Figure 1.3   Chemical structure of salicylic acid.

Jasmonates

Jasmonates, including jasmonic acid (JA) and its pathway intermediates, are important in various plant responses such as plant defense, wound response, pollen

maturation, fruit ripening, root growth, and tendril coiling (Figure 1.4, Turner et al.

2002). The role of jasmonates as a defense mechanism in plants was first suggested by

Farmer and Ryan (1992). They showed that there was a link between wounding by insect

herbivores and the production of jasmonates (Farmer and Ryan 1992).
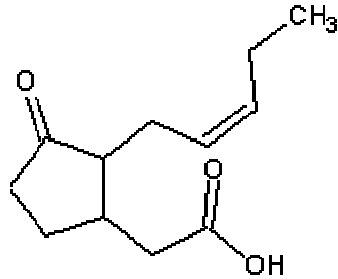
Figure 1.4   Chemical structure of jasmonic acid.

Upon wounding, protosystemin, which is present in low levels in the cytoplasm of

plant cells, is exposed to proteases and is cleaved into systemin by a pathway that is not

fully understood. Systemin interacts with a transmembrane receptor in the cell

membrane, transducing the signal inside the cell. This signal transduction activates

phospholipase A2, which releases linolenic acid from membrane lipids (Gatehouse 2002).

A lipoxygenase (LOX) then oxygenates linolenic acid to its hydroperoxy derivate, 13-

hydroperoxy-octadecatrienoic acid. Allene oxide synthase (AOS) dehydrates 13-

hydroperoxy-octadecatrienoic acid to an unstable epoxide, and allene oxide cyclase

(AOC) catalyzes the cyclization of the allene oxide to (9S,13S)-12 oxo-(10,15Z)-

phytodienoic acid (OPDA). OPDA reductase reduces OPDA to 3-oxo-2-(2'(Z)-

pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). After three rounds of β-oxidation,

OPC-8:0 forms jasmonic acid (Figure 1.5, Turner et al. 2002, Gatehouse 2002,

Halitschke and Baldwin 2004). (Z)-jasmone, created after one additional round of β-

oxidation, is often released from a plant in response to damage (Figure 1.6). Methyl jasmonate, which is formed by the methylation of jasmonic acid by an S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT), is also released as a volatile by plants (Turner et al. 2002).

Plants regulate the synthesis of JA by controlling the transcription of the JA biosynthesis genes that code for the enzymes mentioned previously. A positive feedback system is in place, where the JA biosynthesis genes are upregulated after wounding or treatment with jasmonate (Gatehouse 2002). In addition, JMT is also upregulated upon wounding and in the presence of jasmonate. This causes an increase in methyl jasmonate, which diffuses from the plant and is hypothesized to signal different parts of the same plant, as well as neighboring plants, to the presence of a pest (Gatehouse 2002; Turner et al. 2002).
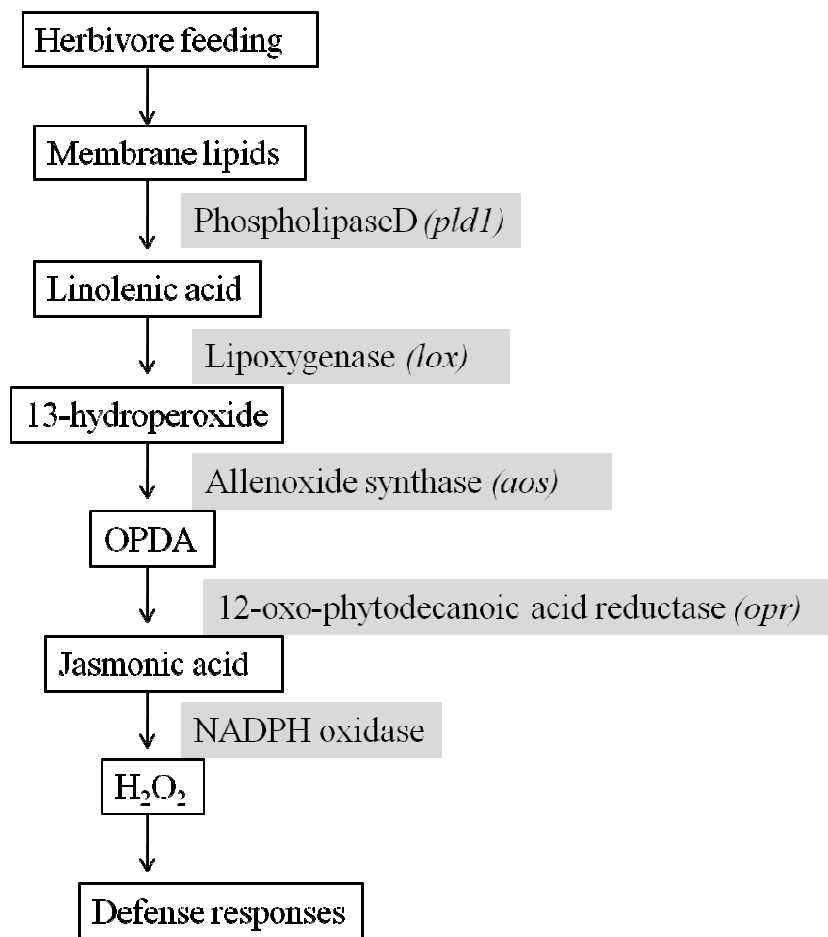
Figure 1.5   Jasmonic acid pathway in plant defense responses.
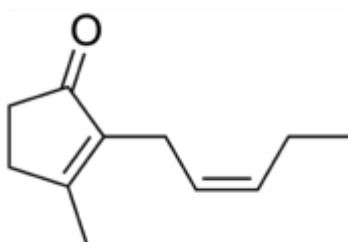
Figure 1.6   Chemical structure of (Z)-jasmone.

Ethylene

Ethylene is a plant hormone that plays an important role in plant development, such as fruit ripening, seed germination, and leaf expansion (Figure 1.7). It has also been

established as a potent activator of plant defense responses (Chang and Shockey 1999). Ethylene is rapidly upregulated upon wounding in plants, due to an increase in the activity of the rate-limiting enzyme in ethylene biosynthesis, S-adenosyl-L-methionine methylthioadenosine-lyase (ACC) synthase (Ecker and Davis 1987). Ethylene gas is then bound by a family of ethylene receptor homodimers. When ethylene is not present, the downstream negative regulator CTR1 is activated and the ethylene response is repressed. When ethylene is present, it binds to the receptors and inhibits the activation of CTR1. The absence of CTR1 activates the carboxy-terminal domain of EIN2, the integral membrane domain of the ethylene receptor, which in turn activates the transcription factor EIN3. EIN3 induces the expression of another transcription factor, ERF1, which binds to the promoter of several genes regulated by ethylene (Chang and Shockey 1999).
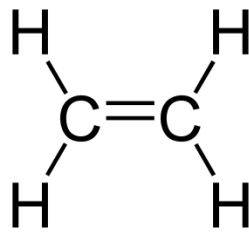
Figure 1.7   Chemical structure of ethylene.

Plant defense response genes regulated by ethylene include L-phenylalanine ammonialyase (PAL) and 4-coumarate:CoA ligase, which are part of the phenylpropanoid pathway; this pathway produces phenolic compounds that are involved in the formation of plant cell walls and antibiotics. Chalcone synthase (CHS), an enzyme involved in the synthesis of an intermediate in the flavanoid and phytoalexin pathways that are commonly induced during wounding, is also upregulated by ethylene (Ecker and Davis 1987). Ethylene works downstream of JA to stimulate the pathway leading to

15

induced systemic resistance (ISR) (Walling 2000). Ethylene, in conjunction with JA, has

been shown to work together to induce osmotin, a common pathogenesis-related (PR)

protein that accumulates in response to infection (Xu et al. 1994).

Abscisic Acid

The plant hormone abscisic acid (ABA), which has long been known to play an

important role in plant development, has recently been implicated in plant defense as well

(Figure 1.8). ABA antagonizes the JA-ethylene pathways; a high concentration of ABA

present for an extended period of time suppresses the transcription of genes involved in

JA-ethylene mediated defense. An ABA deficiency upregulates these same genes

(Anderson et al. 2004). A short-term spike in ABA levels, which occurs in response to

any abiotic stressor, upregulates anti-stress pathways such as the production of callose

(Maksimov 2009).
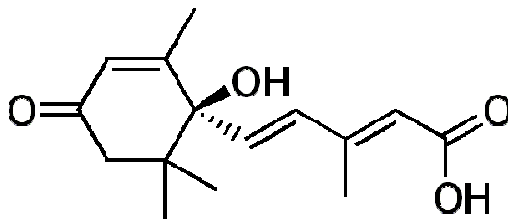
Figure 1.8   Chemical structure of abscisic acid.

The Role of Ubiquitin

The attachment of ubiquitin to a protein is usually a signal that targets that protein

to the proteasome for degradation. However, recent research has suggested that the

ubiquitylation of a protein in fungi and vertebrates does not always lead to protein

degradation. Rather, ubiquitin has also been linked to non-proteasomal functions such as

DNA repair, protein activiation, and ribosomal regulation (Dreher and Callis 2007). The

16

ubiquitylation of transcription factors involved in the ethylene response, for example, allows for the plant cell to maintain a tight control on the amount of ethylene biosynthesis. Recent research has shown that some plant viruses, wounding, JA, SA, and an ethylene precursor all upregulated two enzymes that activate ubiquitin. Thus, plants seem to increase their capability for ubiquitylation when attacked by some sort of pathogen (Dreher and Callis 2007).

The process of *R*-gene-mediated diseases resistance, in which plant resistance (*R*) genes recognize specific pathogen avirulence (*avr*) genes, can also trigger signal transduction pathways that lead to local responses to pathogen attack. Several subunits and regulators of SCF (SKP2/CDC53p/CUL1 F-box) ubiquitin E3 ligases have been shown to be necessary for (*R*)-gene-mediated defense (Devoto et al. 2003).

Interaction of Plant Defense Pathways

In general, biotrophic pathogens elicit a SA-mediated defense pathway, while necrotrophic pathogens and herbivorous insects are more susceptible to the JA/ethylene pathways (Koornneef and Pieterse 2008). These different plant defense pathways do not act alone; rather, they interact to form more complex cascades (Rojo et al. 2003). Ethylene and JA work together to induce the expression of defensin and other pathogenesis-related proteins (Walling 2000, Penninckx et al. 1998). An Arabidopsis mutant in which the JA and ethylene pathways were constitutively expressed showed a higher level of pathogen resistance (Ellis and Turner 2001). On the other hand, SA has been shown to inhibit JA synthesis, and JA is required to induce the conversion of SA to methyl salicylate (Walling 2000, Ament et al. 2004).

17

Volatiles

Plants release volatiles in response to injury by an herbivore or general wounding. Some volatiles are released immediately, within one hour of wounding, and others are synthesized upon wounding and are released five to six hours later. Common volatiles include C6 compounds, indole, methyl salicylate, terpenoids, oximes, and nitriles (Walling 2000). These volatiles attract predators of the herbivore, deter the herbivore from continuing to feed on the plant, and also signal other parts of the plant, as well as neighboring plants, to be on the defensive (Farmer 2001).

(Z)-jasmone is another common volatile released by plants. A recent study of lettuce aphids demonstrated that they were repelled by (Z)-jasmone, but insects antagonistic to the aphids were attracted by (Z)-jasmone (Birkett et al. 2000). It appears that the function of (Z)-jasmone is therefore twofold; it both repels insects that feed on a plant while at the same time attracting other antagonistic insects. Furthermore, the volatile blend released by a plant upon feeding by an herbivore is specific to that herbivore. In studies of *Cardiochiles nigriceps*, a parasitic wasp that feeds specifically on *Heliothis virescens* and not *Helicoverpa zea,* the wasp was able to distinguish between tobacco, cotton, and maize infected with its host, *H. virescens*, and *H. zea*. Even when the damaged portion of the plant was removed, the wasps still picked the correct plant, which was attributed to the different volatile cocktail released from plants infected with *H. virescens* from plants infected with *H. zea* (DeMoraes et al. 1998). Inducing volatile compounds upon attack by pests comes at a cost to the plant, however. Maize plants treated with a regurgitant of *Spodoptera littoralis* for two weeks, eliciting the release of volatiles, had leaves with a lower dry-weight than untreated plants. By maturity, seed

production was no different for treated versus untreated plants, so the maize plants were able to compensate and the effects were not permanent (Hoballah et al. 2004).

Caryophyllene, a Common Plant Volatile

(E)-β-caryophyllene is a volatile commonly emitted by a wide range of plants and insects, from wild maize to female *Harmonia axyridis,* the Asian lady beetle (Kollner et al. 2008, Brown et al. 2006). Caryophyllene is typically released by the roots of many European and wild varieties of maize upon attack by the western corn rootworm; the compound attracts nematodes that are natural enemies of the rootworms (Degenhardt et al. 2009). Most North American varieties of maize retain terpene synthase 23 (TPS23), the gene that produces caryophyllene from farnesyl diphosphate, but it is not actively transcribed (Kollner et al. 2008). Farnesyl diphosphate is synthesized from the condensation of one molecule of dimethylallyl pyrophosphate with two molecules of isopentyl diphosphate, both of which are produced during the mevalonate and methylerythritol phosphate pathways in maize (Figure 1.9, Kappers et al. 2005). Maize plants engineered with the caryophyllene synthase gene from oregano had significantly less root damage when infested with rootworms in the presence of nematodes (Degenhardt et al. 2009). Caryophyllene has also been shown to be released by the leaves of maize plants after feeding by other larvae from the *Spodoptera* genus, and is an attractant to parasitic wasps (Kollner et al. 2008).
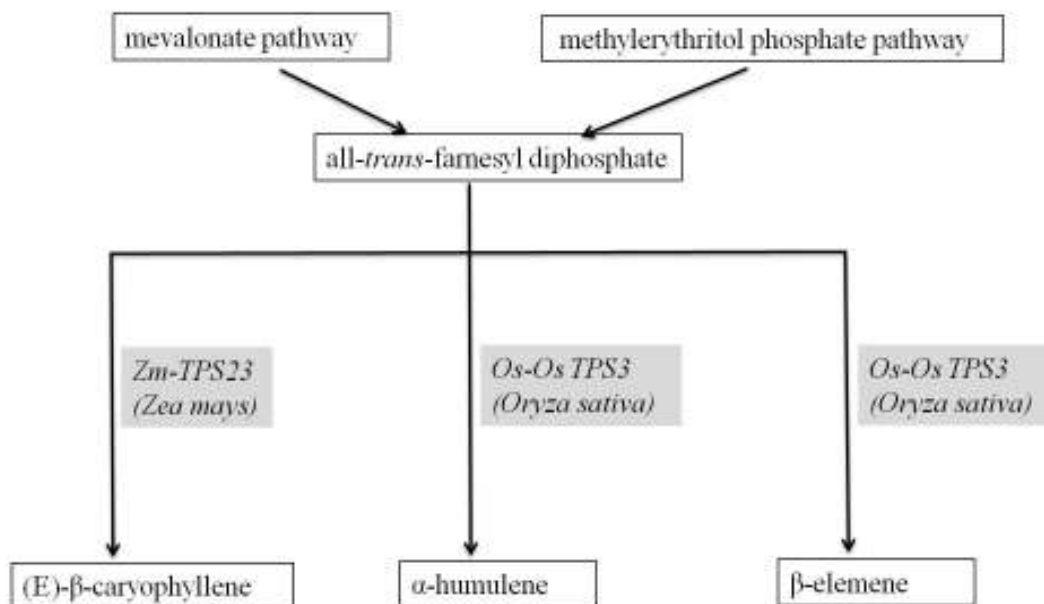
Figure 1.9   Caryophyllene synthesis pathway.

Collection of Plant Volatiles Utilizing SPME

The use of gas chromatography (GC) coupled with mass spectrometry (MS) is a powerful technique for analyzing an unknown mixture of compounds. Gas chromatography is composed of a mobile, inert gas phase and a stationary phase. The stationary phase consists of a glass column containing a support coated with a liquid or polymer. When a mixture of compounds is passed through the GC, they interact with the different stationary phases inside the column. Dissimilar compounds interact differently with the stationary phases, causing them to elute at different times. The time it takes for a specific compound to elute from the GC is the compound's retention time; this retention time is known for most compounds (Silberberg 2003).

Mass spectrometry is a technique for identifying specific compounds based on their mass-to-charge ratio. After compounds are separated using GC, they flow into a mass spectrometer for further analysis. Upon MS entry, compounds are fragmented by

electron impact, forming charged particles. An electrical field is used to separate ions of different mass-to-charge ratios, and then these compounds are detected and analyzed. Every compound has a distinct mass spectrum that can be used for identification (Silberberg 2003). Coupling GC/MS is a powerful technique to identify and analyze compounds from a mixture of unknown volatiles.

The use of solid-phase microextraction (SPME) is a new method for analyzing volatile chemicals. SPME was developed for analyzing pollutants in water samples in the 1990s and has also been used for the analysis of airborne insect pheromones (Brown et al. 2006). Additionally, SPME is being used to look for volatiles produced by toxic *A. flavus* species in maize fields (McDaniel, unpublished data). In this process, a matrix is fused onto a fiber, and volatile compounds are absorbed onto the fiber. The compounds are then desorbed into the heated injection port of the GC. This method does not require lengthy extraction and concentration of volatiles, and the use of an autosampler reduces human input. Samples are simply sealed into vials and the GC absorbs and desorbs automatically (Brown et al. 2006).

Analyzing Genes Involved in Resistance

Polymerase chain reaction (PCR) is one of the most indispensible tools used in the molecular biology lab. While PCR amplifies a gene of interest quickly and efficiently, it is very difficult to quantify the amount of starting material. To quantify a gene, such as one suspected to be involved in resistance of a plant to a herbivore, using PCR, each sample must have equal beginning amounts of nucleic acid and must amplify with equal efficiency. The reaction is then stopped at the log phase and quantified. A more commonly used method is quantitative competitive (QC)-PCR, which includes an

internal control in each reaction that competes with the template for replication. The concentration of the competitor is known, and the unknown PCR product can be compared to it for a relative quantification (Heid et al. 1996).

Quantitative real-time polymerase chain reaction (qRT-PCR) is a rapid and effective method for quantifying DNA sequences. Developed in the 1990s, it allows for the quantification of PCR products as they are being synthesized, i.e. in real time. There are two methods of quantification: probe-based and SYBR Green based. The probe-based method relies on the use of a fluorescent probe labeled with two different dyes. The reporter dye fluoresces when the probe is intact, and this fluorescence is absorbed by a quenching dye. The probe is cleaved during the extension step by the exonuclease activity of the DNA polymerase; when the probe is cleaved, the reporter dye is not efficiently absorbed by the quenching dye, so the reporter dye fluoresces and can be detected. The reporter dye intensity is low during the early cycles, and when enough probe has been cleaved, the intensity increases logarithmically. The cycle number at which the amplification passes an arbitrarily set threshold, usually around 15-20 cycles, is defined as the C_T value. The C_T value decreases linearly as the amount of target is increased and can be used to quantify the number of target DNA in the sample at the beginning of the reaction (Heid et al. 1996). The SYBR Green based method of qRT-PCR utilizes a SYBR Green fluorescent dye that binds to double-stranded DNA only. As more double-stranded DNA products are produced, the SYBR Green dye signal becomes stronger and is detected by the RT-PCR detector (Simpson et al. 2000). If a reverse transcriptase is used to synthesize cDNA from RNA prior to conducting qRT-PCR, the end result is the concentration of transcripts of a gene of interest.

Conclusion

Understanding plant defense pathways is important for the analysis of resistance of maize to the fall armyworm larvae. Volatiles play a major role in the defense mechanisms of sessile organisms such as maize. These volatiles are produced as a result of many different pathways activated by various types of wounding, from mechanical wounding to feeding by insects with sucking and/or tearing mouthparts. The role volatiles play in the resistance of maize to feeding by the fall armyworm larvae is not well understood. Research into this subject provides insights into possible mechanisms of resistance. Volatiles could be released constitutively or upon feeding, repelling the fall armyworm larvae. Different volatiles might be released that attract natural enemies of the fall armyworm larvae. Understanding how the volatile cocktail released by a resistant line of maize interacts with the fall armyworm larvae could provide insights into possible mechanisms of resistance. The identification of these compounds and their underlying genes could be used to engineer fall armyworm resistance into commercial varieties of maize.

References

Ament K., Kant M.R., Sabelis M.W., Haring M.A., Schuurink R.C. (2004) Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. Plant Physiology 135: 2025-2037.

Anderson J.P., Badruzsaufari E., Schenk P.M., Manners J.M., Desmond O.J., Ehlert C., Maclean D.J., Ebert P.R., Kazan K. (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. The Plant Cell 16: 3460–3479.

Birkett M.A., Campbell C.A.M., Chamberlain K., Guerrieri E., Hick A.J., Martin J.L., Matthes M., Napier J.A., Pettersson J., Pickett J.A., Poppy G.M., Pow E.M., Pye B.J., Smart L.E., Wadhams G.H., Wadhams L.J., Woodcock C.M. (2000) New roles for *cis*-jasmone as an insect semichemical and in plant defense. Proc. Natl. Acad. Sci. USA 97: 9329-9334.

Brown A. E. 2006. Mode of Action of Insecticides and Related Pest Control Chemicals for Production Agriculture, Ornamentals, and Turf. Maryland Cooperative Extension. Pesticide Information Leaflet 43.

Brown A.E., Riddick E.W., Aldrich J.R., Holmes W.E. (2006) Identification of (-)-β-caryophyllene as a gender-specific terpene produced by the multicolored Asian lady beetle. Journal of Chemical Ecology 32: 2489-2499.

Catchot A., editor. 2010. Insect Control Guide for Agronomic Crops. Mississippi State University Extension Service. Publication 2471.

Chang C., Shockey J.A. (1999) The ethylene-response pathway: signal perception to gene regulation. Current Opinion in Plant Biology 2: 352-358.

Chang Y.M., Luthe D.S., Davis F.M., Williams W.P. (2000) Influence of whorl region from resistant and susceptible corn genotypes on fall armyworm (Lepidoptera: Noctuidae) growth and development. Journal of Economic Entomology 93: 478-483.

Chen Y., Ni X., Buntin G.D. (2009) Physiological, nutritional, and biochemical bases of corn resistance to foliage-feeding fall armyworm. Journal of Chemical Ecology 35: 297-306.

Chilcutt C.F., Odvody G.N., Correa J.C., Remmers J. (2007) Effects of Bacillus thuringiensis transgenic corn on corn earworm and fall armyworm (Lepidoptera: Noctuidae) densities. Journal of Economic Entomology 100: 327-334.

Chungjatupornchai W., Hofte H., Seurinck J., Angsuthanasombat C., Vaeck M. (1988) Common features of Bacillus thuringiensis toxins specific for Diptera and Lepidoptera. European Journal of Biochemistry 173: 9-16.

Corn Insect Pests: A Diagnostic Guide. (1998) University of Missouri Extension.

Davis F.M., Baker G.T., Williams W.P. (1995) Anatomical characteristics of maize resistant to leaf feeding by southwestern corn borer (Lepidoptera: Pyralidae) and fall armyworm (Lepidoptera: Noctuidae). Journal of Agricultural Entomology 12: 55-65.

Davis F.M., Williams W.P., Chang Y.M., Baker G.T., Hedin P.A. (1999) Differential growth of fall armyworm larvae (Lepidoptera: Noctuidae) reared on three phenotypic regions of whorl leaves from a resistance and a susceptible maize hybrid. Florida Entomologist 82: 248-254.

Degenhardt J., Hiltpold I., Kollner T.G., Frey M., Gierl A., Gershenzon J., Hibbard B.E., Ellersieck M.R., Turlings T.C.J. (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proceedings of the National Academy of Sciences of the USA 106: 13213-13218.

Dreher K., Callis J. (2007) Ubiquitin, hormones and biotic stress inplants. Annals of Botany 99: 787-822.

DeMoraes C.M., Lewis W.J., Pare P.W., Alborn H.T., Tumlinson J.H. (1998) Herbivore-infested plants selectively attract parasitoids. Nature 393: 570-572.

Devoto A., Muskett P.R., Shirasu K. (2003) Role of ubiquitination in the regulation of plant defense against pathogens. Current Opinion in Plant Biology 6: 307-311.

Ecker J.R., Davis R.W. (1987) Plant defense genes are regulated by ethylene. Proc. Natl. Acad. Sci. USA 84: 5202-5206.

Ellis C., Turner J.G. (2001) The Arabidopsis mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. The Plant Cell 13: 1025-1033.

Fall Armyworm Agronomic Spotlight. (2010) Monsanto.

Farmer E.E. (2001) Surface-to-air signals. Nature 211: 854-856.

Farmer E.E., Ryan C.A. (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. The Plant Cell 4: 129-134.

Farrar R.R., Shepard B., Shapiro M., Hassell R.L., Shaffer M.L., Smith C.M. (2009) Supplemental control of lepidopterous pests on Bt transgenic sweet corn with biologically-based spray treatments. Journal of Insect Science 9:8, available online: insectscience.org/09.08.

Gatehouse J.A. (2002) Plant resistance towards insect herbivores: a dynamic interaction. New Phytologist 156: 145-169.

Halitschke R., Baldwin I.T. (2005) Jasmonates and related compounds in plant-insect interactions. Journal of Plant Growth Regulation 23: 238-245.

Heid C.A., Stevens J., Livak K.J., Williams P.M. (1996) Real time quantitative PCR. Genome Research 6: 986-994.

Hoballah M.E., Kollner T.G., Degenhardt J., Turlings T.C.J. (2004) Costs of induced volatile production in maize. Oikos 105: 168-180.

Hofte H., de Greve H., Seurinch J., Jansens S., Mahillon J., Ampe C., Vandekerckhove J., Vanderbruggen H., van Montagu M., ZabeauM., Vaeck M. (1986) Structural and functional analysis of a coloned delta endotoxin of *Bacillus thuringiensis Berliner* 1715. European Journal of Biochemistry 161: 273-280.

Hopkins B. W., Pietrantonio P. V. (2009) Differential efficacy of three commonly used pyrethroids against laboratory and field-collected larvae and adults of Helicoverpa zea (Lepidoptera: Noctuidae) and significance for pyrethroid resistance management. Pest Management Science 66: 147-154.

Kitz R., Wilson I. B. (1962) Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. Journal of Biological Chemistry 237: 3245-3249.

Kollner T.G., Held M., Lenk C., Hiltpold I., Turlings T.C.J., Gershenzon J., Degenhardt J. (2008) A maize (E)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. The Plant Cell 20: 482-494.

Koornneef A., Pieterse C.M.J. (2008) Cross talk in defense signaling. Plant Physiology 146: 839-844.

Loake G., Grant M. (2007) Salicylic acid in plant defense—the players and protagonists. Current Opinion in Plant Biology 10: 466-472.

Maksimov I.V. (2009) Abscisic acid in the plants-pathogen interaction. Russian Journal of Plant Physiology 56: 742-752.

Pechan T., Cohen A., Williams W.P., Luthe D.S. (2002) Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proc. Natl. Acad. Sciences U.S.A. 99: 13319-13323.

Pechan T., Ye L., Chang Y., Mitra A., Lin L., Davis F.M., Williams W.P., Luthe D.S. (2000) A unique 33-kD cysteine proteinase accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other Lepidoptera. The Plant Cell 12: 1031-1040.

Pedigo L.P. Entomology and Pest Management. 4th ed. Prentice Hall: Upper Saddle River, New Jersey, 2002.

Penninckx I.A.M.A., Thomma B.P.H.J., Buchala A., Metraux J.P., Broekaert W.F. (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensis gene in Arabidopsis. The Plant Cell 10: 2103-2113.

Rojo E., Solano R., Sanchez-Serrano J.J. (2003) Interactions between signaling compounds involved in plant defense. Journal of Plant Growth Regulation 22: 82-98.

Shah J. (2003) The salicylic acid loop in plant defense. Current Opinions in Plant Biology 6: 365-371.

Shivaji R., Camas A., Ankala A., Engelberth J., Tumlinson J.H., Williams W.P., Wilkinson J.R., Luthe D.S. (2010) Plants on constant alert: elevated levels of jasmonic acid and jasmonate-induced transcripts in caterpillar-resistant maize. Journal of Chemical Ecology 36: 179-191.

Silberberg M.S. Chemistry: The Molecular Nature of Matter and Change. 3rd ed. McGraw-Hill. New York City, New York, 2003.

Simpson D.A.C., Feeney S., Boyle C., Stitt A.W. (2000) Retinal VEGF mRNA measured by SYBR Green I fluorescence: A versatile approach to quantitative PCR. Molecular Vision 6: 178-183.

Sparks A.N. (1979) A review of the biology of the fall armyworm. The Florida Entomologist 62: 82-87.

Turner J.G., Ellis C., Devoto A. (2002) The jasmonate signal pathway. The Plant Cell Supplement 2002: S153-S164.

USDA, ARS, National Genetic Resources Program. Germplasm Resources Information Network - (GRIN). [Online Database] National Germplasm Resources

Laboratory, Beltsville, Maryland. Available: http://www.ars-grin.gov/cgi-bin/npgs/acc/search.pl?accid=Tx601 (08 September 2010).

Van Rie J., Jansens S., Hofte H., Degheele D., Van Mellaert H. (1989) Specificity of Bacillus thuringiensis δ-endotoxins. European Journal of Biochemistry 186: 239-247.

Walling L. (2000) The myriad plant responses to herbivores. Journal of Plant Growth and Regulation 19: 195-216.

Williams W.P., Buckley P.M., Davis F.M. (1985) Larval growth and behavior of the fall armyworm (Lepidoptera: Noctuidae) on callus initiated from susceptible and resistant corn hybrids. Journal of Economic Entomology 78: 951-954.

Williams W.P., Buckley P.M., Davis F.M. (2000) Vegetative phase change in maize and its association with resistance to fall armyworm. Maydica 45: 215-219.

Williams W.P., Davis F.M., Buckley P.M., Hedin P.A., Baker G.T., Luthe D.S. (1998) Factors associated with resistance to fall armyworm (Lepidoptera: Noctuidae) and southwestern corn borer (Lepidoptera: Crambidae) in corn at different vegetative stages. Journal of Economic Entomology 91: 1471-1480.

Williams W.P., Davis F.M., Windham G.L. (1990) Registration of Mp708 germplasm line of maize. Crop Science 30: 757.

Williams W.P., Davis F.M. (1982) Registration of Mp704 germplasm line of maize. Crop Science 22: 1269-1270.

World of Corn Report. (2009) National Corn Growers Association.

Xu Y., Change P.F.L., Liu D., Narasimhan M.L., Raghothama K.G., Hasegawa P.M., Bressan R.A. (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. The Plant Cell 6: 1077-1085.

CHAPTER II

DETECTION AND CHARACTERIZATION OF A VOLATILE COMPOUND AS A

RESPONSE TO FALL ARMYWORM (*SPODOPTERA FRUGIPERDA)*

IN MAIZE (*ZEA MAYS)*

Abstract

Maize (*Zea mays)* is an important agricultural crop grown in the United States.

Various pests, such as those in the Lepidoptera family, frequently feed on young maize

plants and pose a significant threat to plant development and survival. To deal with this

problem, maize generates a wide variety of responses to attack by pests, from activation

of wound-response pathways such as jasmonic acid (JA) biosynthesis to the release of

volatile compounds. Several maize lines have been developed that show resistance to

one common Lepidoptera pest, the larvae of the fall armyworm (*Spodoptera frugiperda*).

Mp708, an inbred line resistant to feeding by fall armyworm, has been developed through

traditional breeding methods, but its method of resistance is not completely understood.

Mp708 has been shown to have a moderately high constitutive expression of JA and other

octadecanoid compounds prior to infestation by fall armyworm. On the other hand

Tx601, a genotype susceptible to feeding by fall armyworm, activates JA pathway only in

response to feeding, suggesting that Mp708 is "primed" to respond swiftly to an attack.

Current research indicates that fall armyworms show a lack of preference to feeding on

Mp708, leading to the hypothesis that volatiles constitutively released by the plant may

also play an important role in its resistance. Analysis of the volatiles released by the

resistant and susceptible lines in the presence and absence of the fall armyworm was conducted using Solid Phase Microextraction (SPME) in conjunction with gas chromatography mass spectrometry (GC/MS). Caryophyllene, a terpenoid compound, was identified in higher levels in the resistant line than the susceptible line, suggesting that it is constitutively produced by the resistant maize. In addition, four day-old fall armyworm larvae show a marked preference for Mp708 whorl tissue over Tx601 tissue.

Ultimately, identifying specific volatiles correlated with resistance to fall armyworm could lead to the integration of these traits into commercial varieties of maize. Resistance to this pest would allow farmers to spray fewer insecticides, saving time, money, and the environment, and these savings could be passed on to consumers.

Introduction

The larval stage of *Spodoptera frugiperda,* known as the fall armyworm, is a common pest of maize (*Zea mays).* The fall armyworm larvae feed on the whorl region of four to six week old maize plants; they specifically show a preference for the yellow-green whorl region (Chang et al. 2000). The development of host-plant resistance is an important method in the control of herbivores like the fall armyworm. By inducing host-plant resistance in maize, insect control is provided by making the crop unpalatable to the pest and thus preventing the initial feeding of the fall armyworm larvae. Resistant maize could also contain compounds that cause damage or are lethal to the larvae. Two lines of maize resistant to fall armyworm feeding, Mp708 and Mp704, have been developed through traditional plant breeding programs (Williams and Davis 1982, Williams et al. 1990). However, the mechanism of resistance of these two maize lines has not been completely investigated. Seven quantitative trait loci (QTL) have been identified on

chromosomes 1, 5, 7, and 9 in Mp708 associated with fall armyworm resistance. An additional region on chromosome 10 was identified in Mp704, a parent of Mp708 (Brooks et al. 2005 and 2007). A unique defense protein, maize insect resistance 1-cysteine protease (Mir1-CP), was also found to accumulate in the yellow-green whorl tissue of a resistant (Mp704 X Mp707) hybrid after infestation with fall armyworms, but did not accumulate in a susceptible hybrid (Pechan et al. 2000). The protein was determined to damage the peritrophic matrix, which separates the food from the midgut, of the fall armyworm larvae (Pechan et al. 2002).

Jasmonates, including jasmonic acid (JA) and its pathway intermediates, are important in various plant responses such as plant defense, wound response, pollen maturation, fruit ripening, root growth, and tendril coiling (Figures 1.4 and 1.6; Turner et al. 2002). More specifically, jasmonates have been shown to be produced upon wounding by insect herbivores (Farmer and Ryan 1992). Shivaji et al. (2010) demonstrated that Mp708 has a moderately high constitutive expression of JA and other octadecanoid compounds prior to infestation by fall armyworm. On the other hand, Tx601, a genotype susceptible to feeding by fall armyworm, activates the JA pathway only in response to feeding, suggesting that Mp708 is "primed" to respond swiftly to an attack. The constitutive expression of genes induced by JA was also higher in Mp708 than Tx601 (Shivaji et al. 2010).

Another common defense mechanism is the release of volatile compounds by the plant upon attack by an herbivore or general wounding. Some volatiles are released immediately, within one hour of wounding, and others are synthesized upon wounding and are released five to six hours later. Common volatiles include C_6 compounds, indole, methyl salicylate, terpenoids, oximes, and nitriles (Walling 2000). These volatiles attract

31

predators of the herbivore, deter the herbivore from continuing to feed on the plant, and also signal other parts of the plant, as well as neighboring plants, to be on the defensive (Farmer 2001). Maize, in particular, has been shown to emit a cocktail of volatile compounds upon attack by caterpillars such as *Spodoptera littoralis;* jasmonic acid is a key regulator of transcription of genes encoding volatile compounds (Rostas et al. 2008). The particular volatiles emitted from an injured plant can vary depending on the species of herbivore, and even different instars or sexual stages of the same herbivore species (Williams et al. 2005).

Volatile compounds released by maize upon attack by herbivores often attract parasitoids of those pests. Various sesquiterpenes are produced by maize upon attack by Lepidopteran species, including (E)-β-farnesene, (E)-β-bergamotene, and (E)-β-caryophyllene (Schnee et al. 2006, Kollner et al. 2008). TPS10, the terpene synthase gene that produces both (E)-β-farnesene and (E)-β-bergamotene, is regulated at the transcript level in maize. Transformation of *Arabidopsis* with TPS10 results in the release of a high amount of its products, which attracts a parasitoid of Lepidoptera, *Cotesia marginiventris* (Schnee et al. 2006). (Z)-3-hexenyl acetate and linalool have been demonstrated to attract *Campoletis chlorideae,* a parasitoid of *Mythmna separate,* a Lepidopteran pest of maize (Yan et al. 2006). The volatile cocktail emitted by maize both constiuitively and upon infestation with the herbivore *Chilo partellu* has also been shown to attract *Dentichasmias busseolae,* a parasitoid of *C. partellus* (Gohole et al. 2003). In studies of *Cardiochiles nigriceps,* a parasitic wasp that feeds specifically on *Heliothis virescens* and not *Helicoverpa zea,* the wasp was able to distinguish between tobacco, cotton, and maize infected with its host, *H. virescens,* and *H. zea.* Even when the damaged portion of the plant was removed, the wasps still picked the correct plant,

which was attributed to the different volatile cocktail released from plants infected with *H. virescens* that from plants infected with *H. zea* (DeMoraes et al. 1998).

The detection of volatiles using Solid-Phase Microextraction (SPME) fibers coupled with gas chromatography/mass spectrometry (GC/MS) is a relatively fast, sensitive, and reliable method. SPME fibers can be used to analyze an unknown mixture of volatiles, such as those released by Mp708, to determine its components and identify specific compounds associated with resistance. Each SPME fiber is coated with carbonex polydimethlysiloxane, which allows for the absorption of volatile compounds. These compounds are then desorbed into the injection port of a gas chromatogram, where they are separated. The separated compounds are then analyzed and identified using a mass spectrometer coupled to the gas chromatogram. This process is easily automated using an autosampler, allowing several samples to be run sequentially with little user input. This method of volatile detection has been used in a variety of applications such as the detection of volatiles released by insects (Brown et al. 2006).

From these observations, we proposed that some component of the volatile cocktail emitted by Mp708, either constitutively or upon feeding by fall armyworm, plays a role in its observed resistance. We also hypothesized that the release of these volatiles would lead fall armyworms to display a lack of preference for Mp708, especially in the presence of Tx601 as an alternate food source.

Materials and Methods

Growth Conditions for Maize

Mp708 and Tx601 maize seeds were grown in 26.7 x 25.4 cm plastic pots using commercially available potting soil (Miracle Grow). Plants were grown under

greenhouse conditions for five weeks with a maximum daily temperature of 33.1 °C and a minimum night temperature of 26.3 °C. Pots were watered as needed.

Volatile Collection and GC/MS Separation

The yellow-green whorl region of the 5 week old maize plants was excised using scissors and placed in 9 x 8 x 2 cm plastic petri dishes. One sample was left alone, and approximately 10 4-day-old fall armyworms were placed in the petri dish of the other sample. One to two pieces of the corn was weighed to approximately 250 mg and removed after 4 hours and placed into an autosampler vial for absorption by an 85 µm carbonex polydimethlysiloxane fiber (SPME, Supeloco™) and analysis by gas chromatography-mass spectrometry (GC/MS). The fiber was preconditioned for 30 minutes at 250°C, exposed to the headspace for 60 minutes at 25°C, and desorbed for 3 minutes at 200°C into the GC/MS. A Varian Start 3600 GC (Varian Chromatography Systems, Walnut Creek, CA) coupled with the Varian Saturn 2000 GC/MS, was used for identification of volatile compounds using the NIST library. In the GC, helium was used as a carrier gas to transmit samples through a Phenomenex ZB5 (30 m x 0.25 mm, with a 0.25 m film) column. For MS analysis, ions with an m/z of 50 to 300 were scanned for 35 minutes at 0.75 seconds per scan. Both electron impact ionization (EI) and chemical ionization (CI) programs were run. To verify the retention time of caryophyllene, 1 µl of a (-)-trans-caryophyllene standard (Sigma) was absorbed onto a KimWipe (KimTech Science), sealed into an autosampler vial, and exposed to the SPME fiber. Relative caryophyllene levels were analyzed using ANOVA with an α=0.05 via Statistical Analysis Software (SAS).

Preference Study

The yellow-green whorl region of the 5 week old maize plants was excised using scissors and cut into 1-inch segments. A set of 30 9 x 8 x 2 cm petri dishes were prepared with one cut segment of Tx601 and one cut segment of Mp708 placed on opposite sides of each petri dish. A single 4-day-old fall armyworm larva was placed on the Tx601 leaf segment in 50% of the petri dishes and a single larvae was placed on the Mp708 leaf segment in the remaining 50% of the petri dishes. After 7, 24, and 48 hours the position of the fall armyworm larvae—either on Tx601, Mp708, or neither—was determined. This experiment was repeated 4 times for the 7 and 24 hour feeding time points and was repeated 3 times for the 48 hour feeding time point. Results were analyzed using ANOVA with an $\alpha=0.05$ via Statistical Analysis Software (SAS).

Results

SPME Fiber Analysis

Total ion chromatograms of volatiles emitted by the excised whorl regions of Mp708 and Tx601 showed a marked difference (Figure 2.1). The total ion chromatograms from Mp708 alone and Mp708 with fall armyworms after 4 hours (Figure 2.2) were similar, as were the chromatograms from Tx601 with and without fall armyworms (Figure 2.3). The presence of a peak with a retention time of 12 seconds was noted in the Mp708 but not Tx601 volatile cocktail (Figures 2.5 and 2.6). This peak was identified as caryophyllene, a terpenoid compound that is often a component of plant volatiles (Figure 2.4). An extracted ion chromatogram of ions 133 and 161, found in caryophyllene, shows the presence of caryophyllene in Mp708 alone both with and without FAW feeding (Figure 2.5). However, it was present in very low levels in both

35

Tx601 samples (Figure 2.6). A caryophyllene standard verified the retention time of 11.9-12.1 minutes.
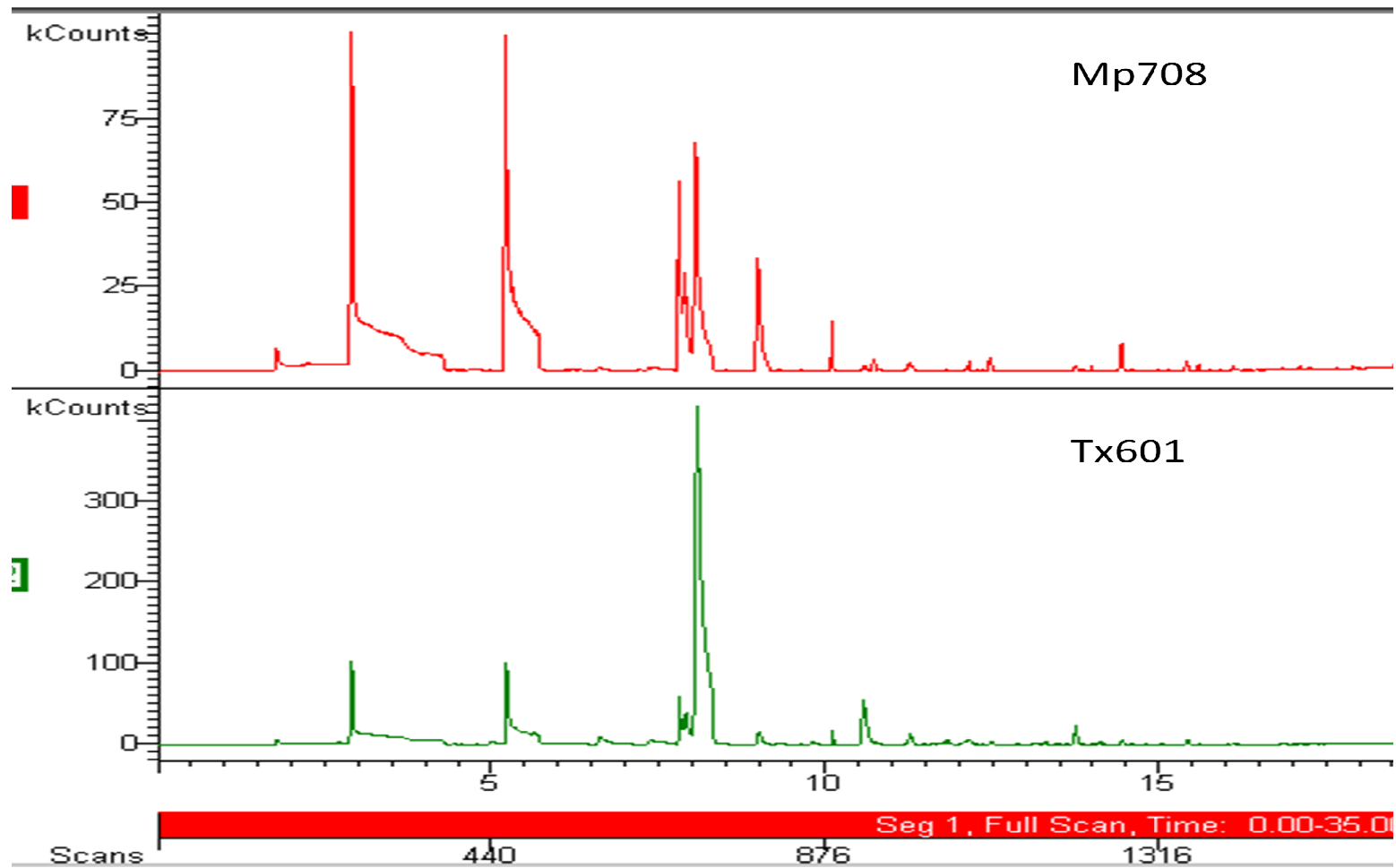
Figure 2.1   Total ion chromatogram of excised whorl tissue from Mp708 and Tx601. The volatile cocktail released is unique to each line.
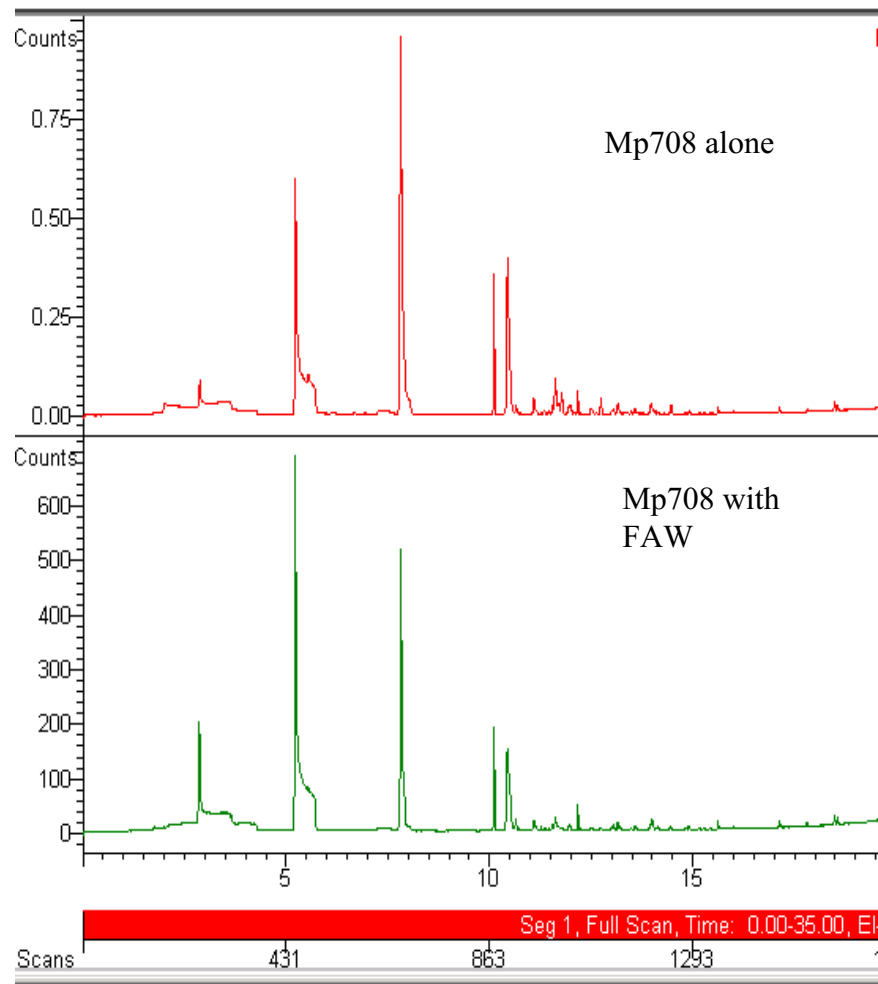
Figure 2.2   Total ion chromatogram of Mp708 alone and Mp708 with fall armyworms.
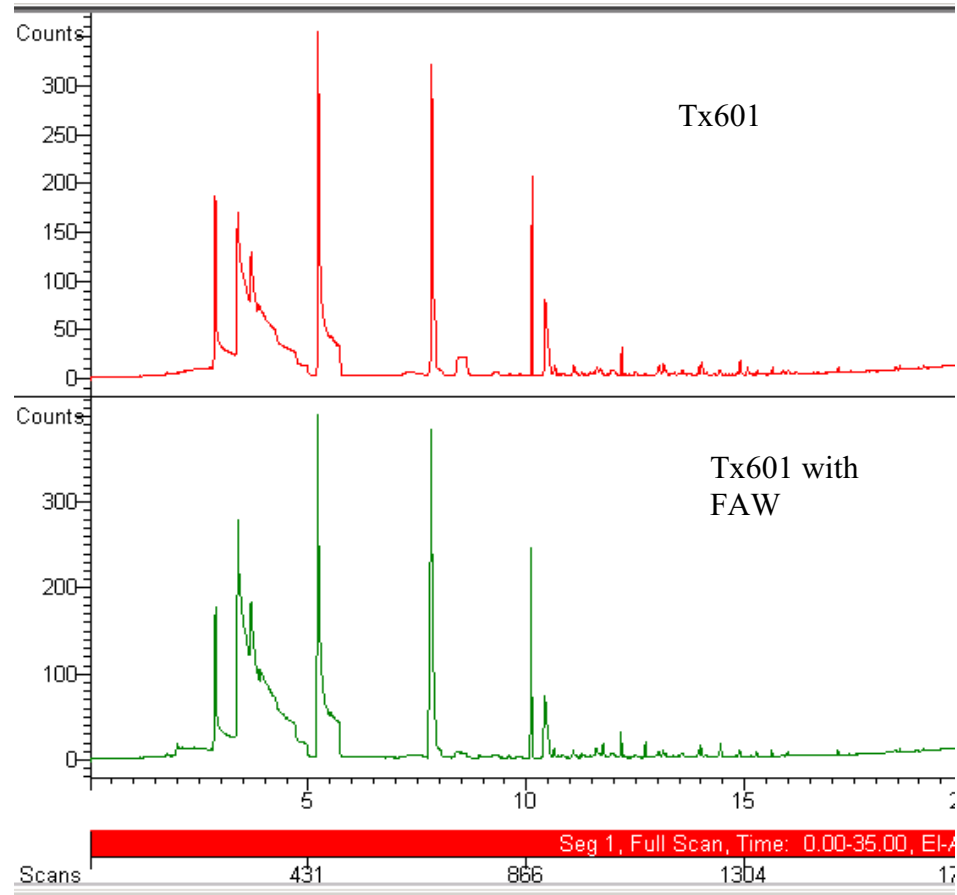
Figure 2.3   Total ion chromatogram of Tx601 alone and Tx601 with fall armyworms.
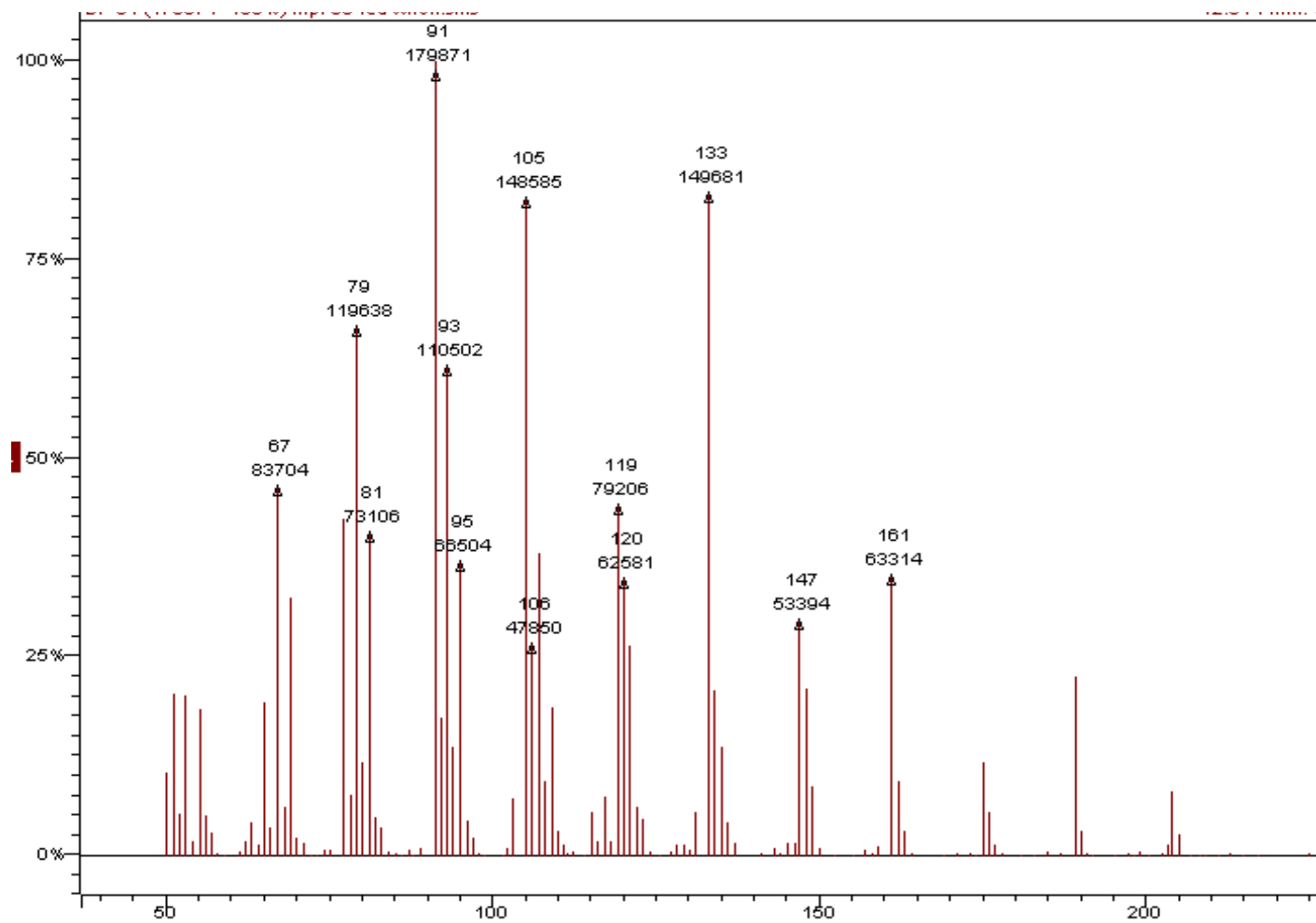
Figure 2.4   Mass spectrum of caryophyllene.
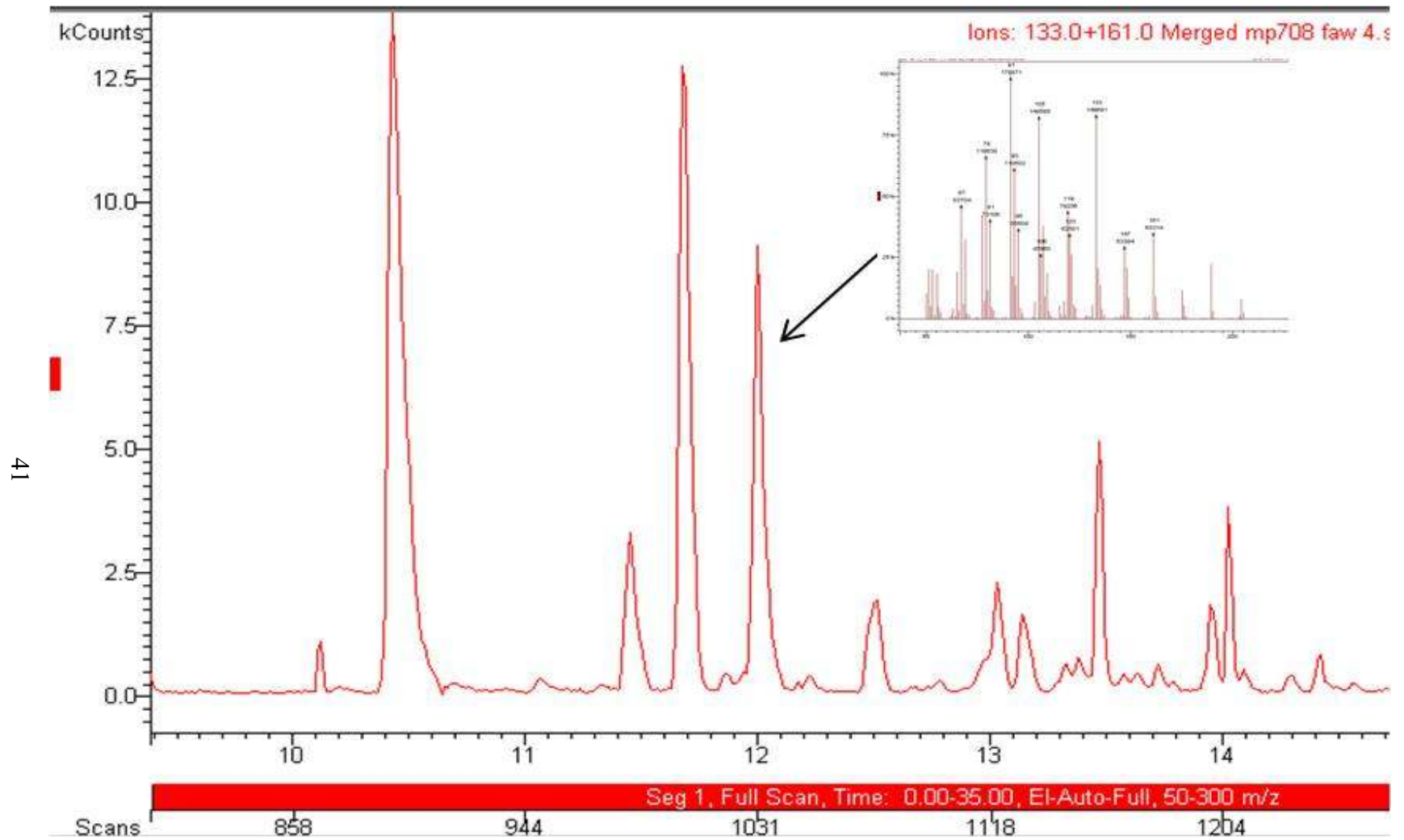
Figure 2.5   Mp708 extracted ion chromatogram showing the presence of a caryophyllene peak eluting at approximately 12 seconds.
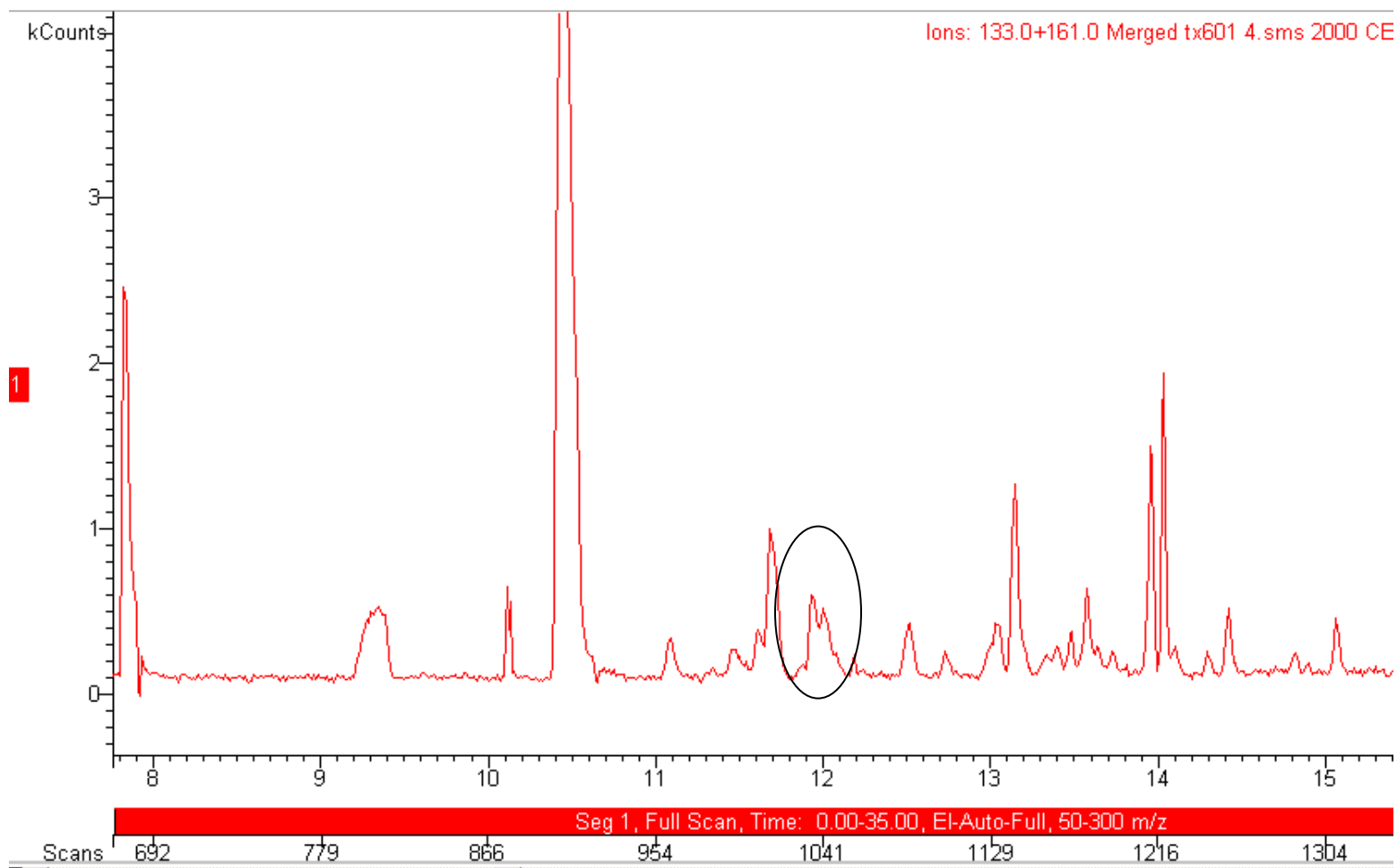
41

Figure 2.6   Extracted ion chromatogram of Tx601 whorl tissue showing the lack of a defined peak eluting at 12 seconds.

Relative caryophyllene levels were monitored in five separate experiments at the 4 hour time point (Table 2.1). While there was variation between experiments, the overall trend suggests that caryophyllene is always present in the volatiles released by Mp708 yellow-green whorl tissue in the presence and absence of FAW. In Tx601, caryophyllene was detected in very small quantities or not at all (Figure 2.5). Overall, the caryophyllene levels in Tx601 were significantly lower than the caryophyllene levels in Mp708 (α=0.05), but feeding by larvae did not appear to produce significant changes in the levels in either the resistant or susceptible line. Analysis of intact Mp708 and Tx601 corn plants shows similar results (Table 3.3) Several commercial maize hybrids, as well as Mp704, a parent of Mp708, were also tested for the presence of caryophyllene. Caryophyllene was not detected or was detected in very low levels in all but one of the commercial lines tested, and was not detected in Mp704 (Table 2.2).

Table 2.1   Relative caryophyllene levels (in kCounts) present in Mp708 and Tx601 yellow-green whorl tissue after either 4 hours alone or in the presence of FAW larvae.

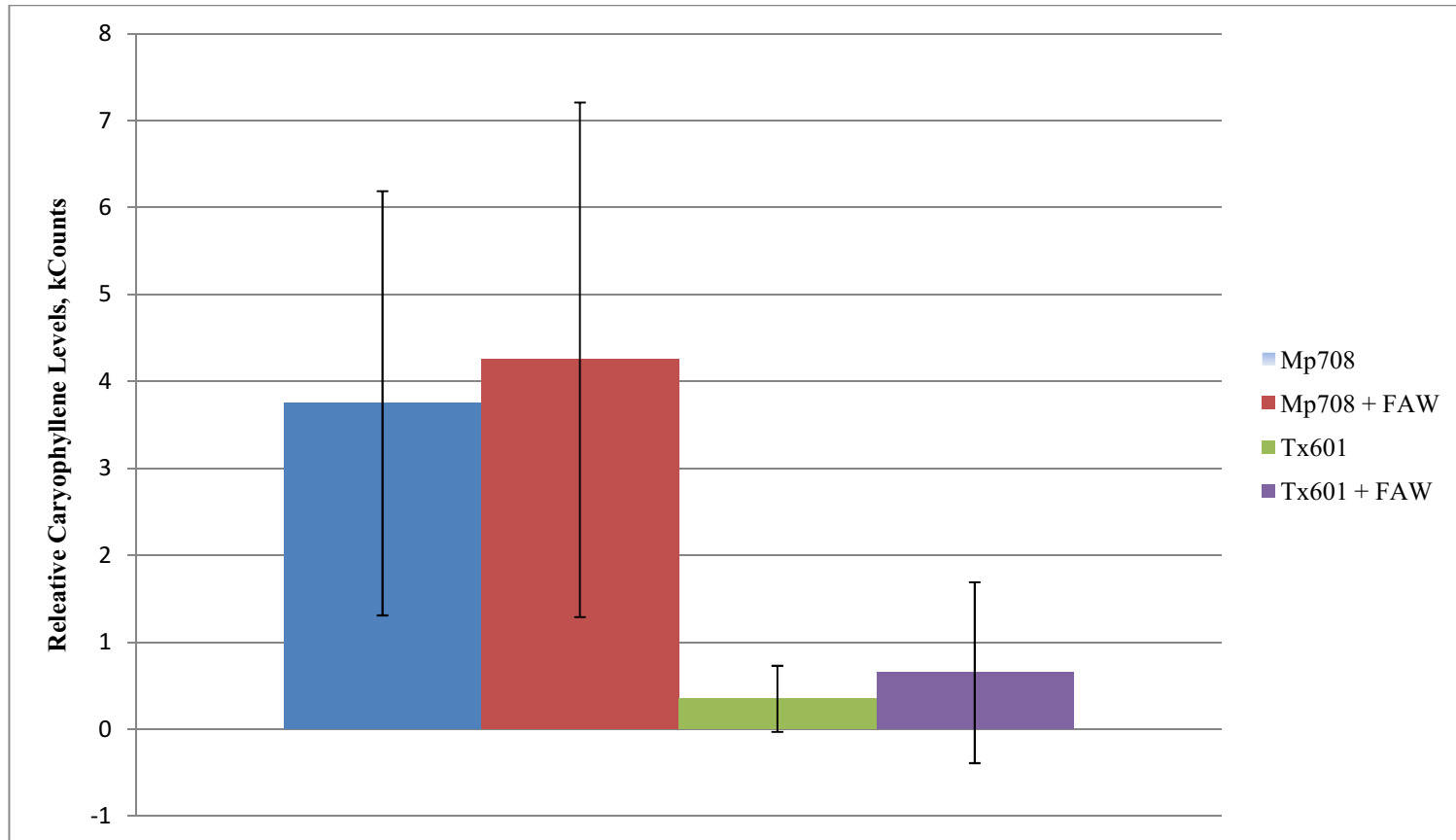| Variety	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Mp708	5	1	4	1.75	7
Mp708 + FAW	4	1	7.5	1.75	7
Tx601	<1	1	<1	<1	n.d
Tx601 + FAW	<1	2.5	<1	<1	n.d

n.d = not detected; FAW = fall armyworm

Figure 2.7   Mean relative caryophyllene levels (with standard deviations) detected in the volatiles of Mp708 and Tx601. The level of caryophyllene in the fed and unfed Mp708 were significantly higher than the level of caryophyllene in fed and unfed Tx601.

Table 2.2   Relative caryophyllene levels detected in Mp704, a breeding parent of
            Mp708, and several commercial maize hybrids. Whorl tissue was collected
            and the caryophyllene levels determined after 1.5 hours in the presence and
            absence of FAW larvae.

Variety	1.5 hr, alone	1.5 hr + FAW
Mp704	n.d.	n.d.
Cropland 6150	n.d.	n.d.
DKC 67-88	2	2
Pioneer P33F85	0.25	n.d.
Cropland 6831	20	20
TV 25R31	0.3	1

n.d. = not detected; FAW = fall armyworm

Preference Study

The results of the preference study are summarized in Figure 2.6. After 7 hours, an average of 7 larvae were located on Mp708, 20.5 had moved to Tx601, and 2.5 larvae were on neither. At the 24 hour time point, 5.75 larvae were on Mp708, 23 were on Tx601, and 1.5 were on neither Mp708 or Tx601. After 48 hours, 3 larvae were on Mp708, 24.7 on Tx601, and 2.3 on neither. The number of FAW larvae that preferred Tx601 was significantly larger ($\alpha = 0.05$) than the number that preferred Mp708 after 7, 24, and 48 hours of feeding. There was no significant difference among the three time points of Mp708 or Tx601.
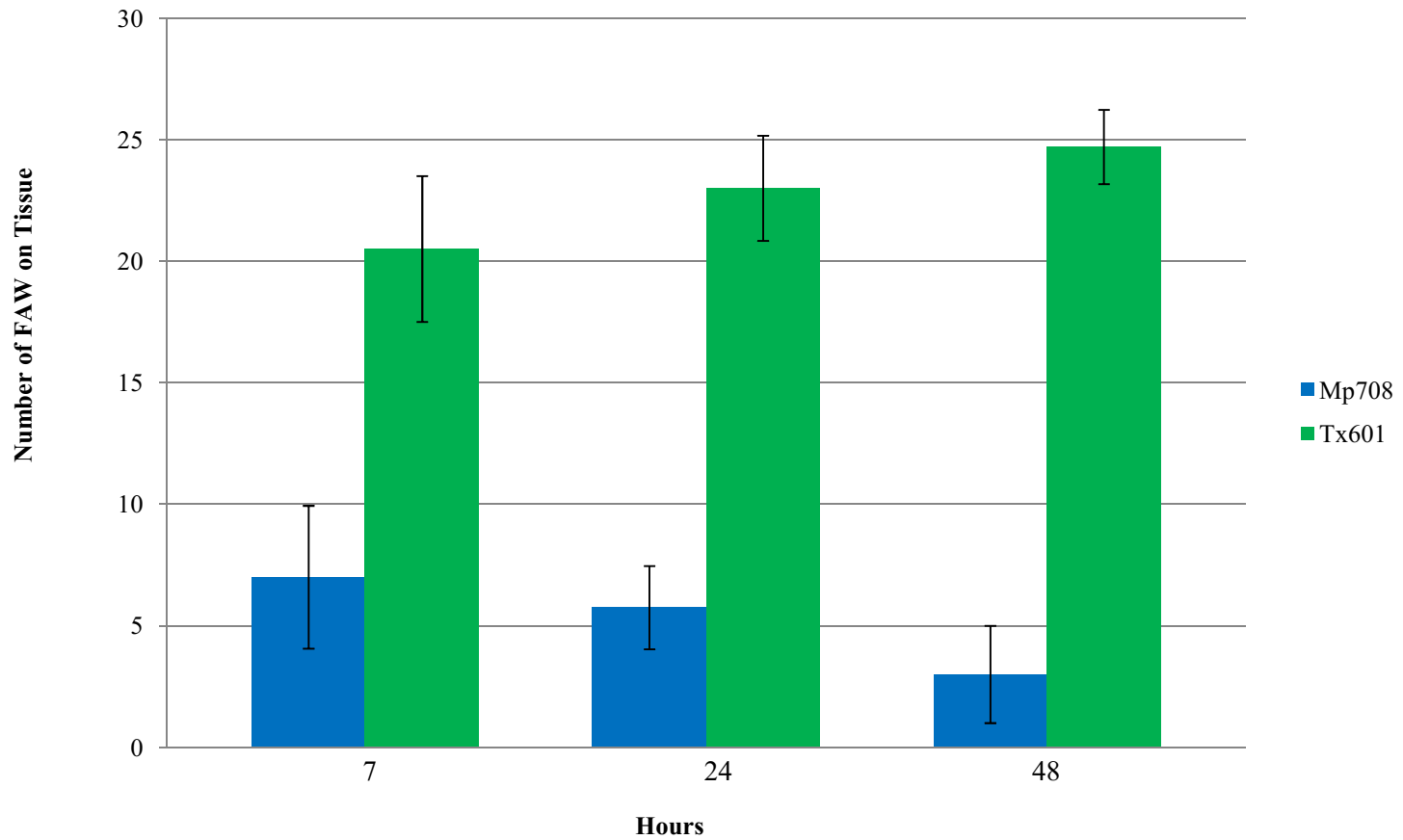
Figure 2.8   Results of preference study using excised Mp708 and Tx601 yellow-green whorl tissue with standard deviations. Significantly more worms chose to feed on Tx601 than Mp708 after 7, 24, and 28 hours.

Discussion

The total ion chromatograms of Mp708 and Tx601 are quite different, providing a good indication that the volatile cocktails released by these two lines are unique (Figure 2.1). Though there are different scales used for the quantities of volatiles present in the Mp708 and Tx601 chromatograms shown in Figure 2.1, the overall chromatograms are still relatively different. Tx601, the resistant line, shows a strong peak with a retention time of 8 minutes that is not present in Mp708. Mp708 has a caryophyllene peak at 12 minutes that is present at a much lower intensity in Tx601. Volatile compounds, unique to each line, are released from the maize plants. These volatiles are not dependent upon FAW feeding but can be produced during normal plant growth. It is very likely that specific components of the volatile cocktail may be responsible for the resistance of Mp708. For example, Mp708 may emit compounds constitutively that repel FAW larvae, or they may release compounds after FAW feeding that deter continued feeding. Alternately, Mp708 may not release volatiles that typically attract FAW larvae. It is probable that both of these mechanisms are at work; for example, Mp708 may release some volatiles that repel FAW larvae while at the same time lacking volatiles that typically attract FAW larvae.

The identification of caryophyllene in the volatiles released by Mp708, and its relative absence in the volatiles of Tx601, provide evidence of a specific compound that may play a role in the resistance of Mp708. However, it is important to note that caryophyllene is still present in small quantities in Tx601. This could be attributed to the fact that both Mp708 and Tx601 were mechanically wounded prior to volatile collection by the excising of the whorl tissue. This mechanical wounding would also trigger a

defense pathway by the plant, possibly releasing a small amount of caryophyllene and other plant volatiles. In addition, preliminary results show that intact Mp708 plants also release caryophyllene. Thus, there is a possibility that Mp708 releases caryophyllene constitutively, while Tx601 does not.

Caryophyllene is a terpenoid compound that is a commonly released plant volatile. It is frequently found in the essential oils distilled from many common plants, and has even been identified as a potential insecticide targeted toward adult mosquitoes (Dua et al. 2010). Caryophyllene is known to attract natural enemies of maize herbivores, and is synthesized from farnesyl diphosphate by terpene synthase 23 (TPS23) (Kollner et al. 2008). Farnesyl diphosphate is synthesized from the condensation of one molecule of dimethylallyl pyrophosphate with two molecules of isopentyl diphosphate, both of which are produced during the mevalonate and methylerythritol phosphate pathways in maize (Kappers et al. 2005).

Caryophyllene synthesis is upregulated by maize upon attack by two insects with piercing-sucking mouthparts, *Lygus hesperus* and *Nezara viridula* (Williams et al. 2005). Caryophyllene has also been identified as the compound emitted by maize roots upon attack by the western corn rootworm; it attracts a nematode enemy of the rootworm (Degenhardt et al. 2009). It is also released by maize leaves and has been shown to attract an entomopathogenic nematode, *Heterorhabditis megidis,* and a parasitic wasp, *Cotesia marginiventris,* members of two common classes of herbivore enemies (Kollner et al. 2008). *C. marginiventris* is also a known parasite of fall armyworm larvae (Ferkovich et al 1983). While most commercial North American varieties of maize contain the gene encoding TPS23, its decreased transcription leads to decreased caryophyllene production. This is verified by SPME fiber analysis of several commercial

maize hybrids available in the Southern U.S., the majority of which show little to no

caryophyllene released (Table 2.2). However, this gene is active in wild maize species

(teosinte) and European maize lines (Kollner et al. 2008). In addition, the TPS23 gene

maps to chromosome 10 in maize; previous analysis has identified a QTL region

associated with resistance to the fall armyworm larvae on chromosome 10 of Mp704, a

parent of the variety used in this study, Mp708 (Brooks et al. 2007).

Maize plants that do not release caryophyllene have been genetically engineered

with a caryophyllene gene from oregano (Degenhardt et al. 2009). This restores their

ability to produce and release caryophyllene, resulting in a 60% reduction in the amount

of adult western corn rootworm beetles that emerged from genetically modified plants

(Degenhardt et al. 2009). Since the breeding parents of Mp704 and Mp708 are tropical in

origin, this suggests that this ability to transcribe TPS23 is active in Mp708 and thus

caryophyllene is present in greater quantities. Since caryophyllene has been identified as

an attractant to natural enemies of the fall armyworm larvae, such as *C. marginiventris,* it

is likely that this mechanism plays a role in the resistance of Mp708 (Kollner et al 2008).

Research examining the use of natural enemies for control of the fall armyworm larvae is

limited, and this appears to be a promising path to pursue in further research.

Previous studies have shown that the FAW larvae display a preference for callus

of susceptible genotypes over resistant genotypes (Williams et al. 1985). In addition,

larvae reared on diet of Mp708 tissue are smaller than those reared on Sc229, a

susceptible line (Chang et al. 2000). This research marks the first time that preference of

Tx601 over Mp708 has been established using the yellow-green whorl region of the

maize plant. The yellow-green whorl region is the preferred feeding site of FAW larvae;

this study provides statistical verification that the FAW larvae prefer the whorl tissue of Tx601 over Mp708.

In conclusion, the identification of caryophyllene, a volatile compound released almost exclusively by a line of maize resistant to the fall armyworm larvae, is an important step in understanding the resistance of Mp708. While this compound was identified only in maize, it is likely that it could be identified in other host-plant interactions as well. Further study elucidating the interactions between caryophyllene, fall armyworm larvae, and natural enemies of the fall armyworm could provide additional insights into the role of caryophyllene in resistance. Since engineering TSP23, the gene involved in the caryophyllene pathway, into maize has been previously accomplished, the insertion of this gene into commercial maize varieties could provide another mechanism of resistance to Lepidopteran pests.

References

Brooks T.D., Bushman B.S., Williams W.P., McMullen M.D., Buckley P.M. (2007) Genetic basis of resistance to fall armyworm (Lepidoptera: Noctuidae) and southwestern corn borer (Lepidoptera: Crambidae) leaf-feeding damage in maize. Journal of Economic Entomology 100: 1470-1475.

Brooks T.D., Willcox M.C., Williams W.P., Buckley P.M. (2005) Quantitative trait loci conferring resistance to fall armyworm and southwestern corn borer leaf feeding damage. Crop Science 45: 2430-2434.

Brown A.E., Riddick E.W., Aldrich J.R., Holmes W.E. (2006) Identification of (-)-β-caryophyllene as a gender-specific terpene produced by the multicolored Asian lady beetle. Journal of Chemical Ecology 32: 2489-2499.

Chang Y.M., Luthe D.S., Davis F.M., Williams W.P. (2000) Influence of whorl region from resistant and susceptible corn genotypes on fall armyworms (Lepidoptera: Noctuidae) growth and development. Journal of Economic Entomology 93: 477-483.

DeMoraes C.M., Lewis W.J., Pare P.W., Alborn H.T., Tumlinson J.H. (1998) Herbivore-infested plants selectively attract parasitoids. Nature 393: 570-572.

Degenhardt J., Hiltpold I., Kollner T.G., Frey M., Gierl A., Gershenzon J., Hibbard B.E., Ellersieck M.R., Turlings T.C.J. (2009) Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. Proceedings of the National Academy of Sciences of the USA 106: 13213-13218.

Dua V.K., Pandey A.C., Dash A.P. (2010) Adulticidal activity of essential oil of Lantana cámara leaves against mosquitoes. Indian Journal of Medical Research 131: 434-439.

Farmer E.E., Ryan C.A. (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. The Plant Cell 4: 129-134.

Ferkovich S.M., Greany P.D., Dillard C. (1983) Changes in haemolymph proteins of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), associated with parasitism by the braconid parasitoid *Cotesia marginiventris* (Cresson). Journal of Insect Physiology 29: 933-942.

Gohole L.S., Overholt W.A., Khan Z.R., Vet L.E.M. (2003) Role of volatiles emitted by host and non-host plants in the foraging behavior of *Dentichasmias busseolae,* a pupal parasitoid of the spotted stemborer *Chilo partellus.* Entomologia Experimentalis et Applicata 107: 1-9.

Kappers IF, Aharoni A, van Herpen TW, Luckerhoff LL, Dicke M, Bouwmeester HJ. (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis. Science 309: 2070-2072.

Kollner T.G., Held M., Lenk C., Hiltpold I., Turlings T.C.J., Gershenzon J., Degenhard J. (2008) A maize (E)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. The Plant Cell 20: 482-494.

Pechan T., Cohen A., Williams W.P., Luthe D.S. (2002) Insect feeding mobilizes a unique plant defense protease that disrupts the peritrophic matrix of caterpillars. Proc. Natl. Acad. Sciences U.S.A. 99: 13319-13323.

Pechan T., Ye L., Chang Y., Mitra A., Lin L., Davis F.M., Williams W.P., Luthe D.S. (2000) A unique 33-kD cysteine proteinase accumulates in response to larval feeding in maize genotypes resistant to fall armyworm and other Lepidoptera. The Plant Cell 12: 1031-1040.

Roastas M., Turlings T.C.J. 2008. Induction of systemic acquired resistance in Zea mays also enhances the plant's attractiveness to parasitoids. Biological Control 46: 178-186.

Schnee C., Kollner T.G., Held M., Turlings T.C.J., Gershenzon J., Degenhardt J. (2006) The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. Proc. Natl. Acad. Sciences U.S.A. 103: 1129-1134.

Shivaji R., Camas A., Ankala A., Engelberth J., Tumlinson J.H., Williams W.P., Wilkinson J.R., Luthe D.S. (2010) Plants on constant alert: Elevated levels of jasmonic acid and jasmonate-induced transcripts in caterpillar-resistant maize. Journal of Chemical Ecology 36: 179-191.

Turner J.G., Ellis C., Devoto A. (2002) The jasmonate signal pathway. The Plant Cell Supplement 2002: S153-S164.

Walling L. (2000) The myriad plant responses to herbivores. Journal of Plant Growth and Regulation 19: 195-216.

Williams L., Saona-Rodriquez C., Pare P.W., Crafts-Brandner S.J. (2005) The piercing-sucking herbivores Lygus hesperus and Nezara viridula induce volatile emissions in plants. Archives of Insect Biochemistry and Physiology 58: 84-96.

Williams W.P., Buckley P.M., Davis F.M. (1985) Larval growth and behavior of the fall armyworm (Lepidoptera: Noctuidae) on callus initiated from susceptible and resistant corn hybrids. Journal of Economic Entomology 78: 951-954.

Williams W.P., Davis F.M. (1982) Registration of Mp704 germplasm line of maize. Crop Science 29: 913-915.

Williams W.P., Davis F.M., Windham G.L. (1990) Registration of Mp708 germplasm line of maize. Crop Science 30: 757.

Yan Z.G., Wang C.Z. (2006) Identification of *Mythmna separata*-induced maize volatile synomones that attract the parasitoid *Campoletis chlorideae.* Journal of Applied Entomology 130: 213-219.

CHAPTER III

EVALUATION OF CARYOPHYLLENE LEVELS IN MAIZE *(ZEA MAYS*)

RESISTANT TO THE FALL ARMYWORM *(SPODOPTERA*

FRUGIPERDA) AND THE EFFECT OF

CARYOPHYLLENE ON FALL

ARMYWORM PREFERENCE

Introduction

Caryophyllene is a terpenoid compound that is often released by plants. In maize (*Zea mays*), caryophyllene has been found to play a role in resistance to the larvae of the fall armyworm (FAW, *Spodoptera frugiperda*). Mp708, a resistant line, emits caryophyllene in the presence and absence of the fall armyworm. Tx601, a susceptible line, does not emit caryophyllene or does so in small quantities. The relative caryophyllene levels in the volatiles were recorded in both lines in the presence and absence of fall armyworm larvae over a period of 8 hours. Mp708 consistently released more caryophyllene than Tx601, but there was no pattern during the time course. Data collected from a wild fall armyworm infestation, as well as Mp708 and Tx601 grown under field conditions, also verified that caryophyllene is produced in much larger quantities in Mp708 than Tx601. In addition, the role of caryophyllene in the preference of the fall armyworm larvae for the susceptible over resistant line was investigated. The presence of pure caryophyllene did not appear to repel the fall armyworms, so it is likely that it does not play a direct role in preference. However, as caryophyllene has been well

established as an attractant of natural enemies of pests, and not a repellent to the pest itself, these findings are consistent with the current understanding of the role of caryophyllene in pest control.

Materials and Methods

Growth Conditions for Maize

Mp708 and Tx601 maize seeds were grown in 26.7 x 25.4 cm plastic pots using commercially available potting soil (Miracle Grow). Plants were grown under greenhouse conditions for five weeks with a maximum daily temperature of 33.1 °C and a minimum night temperature of 26.3 °C. Pots were watered as needed.

Volatile Collection and GC/MS Separation

The yellow-green whorl region of the 5 week old maize plants was excised using scissors and placed in 9 x 8 x 2 cm plastic petri dishes. One sample was left alone, and approximately 10 4-day-old fall armyworms were placed in the petri dish of the other sample. One to two pieces of the corn was weighed to approximately 250 mg and taken out after 1.5, 4, and 8 hours and placed into an autosampler vial for absorption by an 85 μm carbonex polydimethlysiloxane fiber (SPME, Supeloco™) and analysis by gas chromatography-mass spectrometry (GC/MS). The fiber was preconditioned for 30 minutes at 250°C, exposed to the headspace for 60 minutes at 25°C, and desorbed for 3 minutes at 200°C into the GC/MS. A Varian Start 3600 GC (Varian Chromatography Systems, Walnut Creek, CA) coupled with the Varian Saturn 2000 GC/MS, was used for identification of volatile compounds using the NIST library. In the GC, helium was used as a carrier gas to transmit samples through a Phenomenex ZB5 (30 m x 0.25 mm, with a 0.25 m film) column. For MS analysis, ions with an m/z of 50 to 300 were scanned for

35 minutes at 0.75 seconds per scan. Both electron impact ionization (EI) and chemical ionization (CI) programs were run. To verify the retention time of caryophyllene, 1 µl of a (-)-trans-caryophyllene standard (Sigma) was absorbed onto a KimWipe (KimTech Science), sealed into an autosampler vial, and exposed to the SPME fiber. Relative caryophyllene levels were analyzed using ANOVA with Fishers Protected LSD with an α=0.05 via Statistical Analysis Software (SAS).

Quantitative Real-Time PCR (qtRT-PCR)

Total RNA was extracted from Mp708 and Tx601 yellow-green whorl samples during several time points using the BioRad Aurum Total RNA Fatty and Fibrous Extraction Kit. The total RNA was used as a template for first-strand cDNA synthesis utilizing Thermoscript Reverse Transcriptase (Invitrogen).

Primers were designed to amplify a 200-300 base pair fragment from the TPS23 gene in maize. The maize TPS23 sequence was identified using the NCBI database and primers were designed. The sequence of the upper primer was 5' AGTACAGGCCAGGCAATTCATCTCA 3'. The sequence of the lower primer was 5' TGCATCTCCACCATCCTATCTCGT 3'. Primers were verified using traditional PCR with Mp708 and Tx601 total cDNA as a template. TPS23 fragments were ligated into the pGEM-T Easy plasmid and cloned into competent *E. coli* cells (Zymo). These plasmids were used as templates to construct a standard curve. The maize ubiquitin gene was also amplified, ligated into the pGEM-T Easy plasmid, and used for normalization. RT-PCR was carried out using the Roche Light Cycler 480 RT-PCR system and SYBR-Green dye.

Caryophyllene Preference Study

Two 1 inch pieces of yellow-green whorl tissue excised from 5-week old Tx601 plants were placed on opposite sides of a 9 x 8 x 2 cm petri dish. In each plate, one piece of tissue received 10 µl of 100X caryophyllene diluted in hexane, and the other piece of tissue received 10 µl of hexane. A total of 20 plates were placed under a hood and the liquid was allowed to evaporate for 20 minutes. One fall armyworm was then placed on the tissue with caryophyllene on 50% of the plates, and one 5 to 6 day old fall armyworm was placed on the tissue with hexane on the other half of the plates. The plates were put in the dark, and the location of the fall armyworm was observed after 8, 24, and 48 hours.

Results

SPME Fiber Analysis

Table 3.1 Relative caryophyllene levels detected in volatiles emitted by Mp708 after 1.5, 4, and 8 hours in the presence and absence of FAW.

Variety	Rep 1	Rep 2 (field samples)	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7
Mp708 1.5 hr	0.3	2.5	3	0.25-0.5	2.5	3	7.5
Mp708 + FAW 1.5 hr	1.25	3.5-4	7.5	1-1.5	20	3	5
Mp708 4 hr	n/a	n/a	5	1	4	1.75	7
Mp708 + FAW 4 hr	n/a	n/a	4	1	7.5	1.75	7
Mp708 8 hr	n/a	n/a	3	5	5	2.5	3.5
Mp708 + FAW 8 hr	n/a	n/a	3	2.5	2.5	2.5	1

n/a = sample not taken; FAW = fall armyworm

Table 3.2   Relative caryophyllene levels detected in volatiles emitted by Tx601 after
            1.5, 4, and 8 hours in the presence and absence of FAW.

Variety	Rep 1	Rep 2 (field samples)	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7
Tx601 1.5 hr	0.3	0.8-0.9	1.5	0.5	<1	<1	n.d
Tx601 + FAW 1.5 hr	0.25-0.5	0.5-1	0.5	1	<1	4	n.d
Tx601 4 hr	n/a	n/a	<1	1	<1	<1	n.d
Tx601 + FAW 4 hr	n/a	n/a	<1	2.5	<1	<1	n.d
Tx601 8 hr	n/a	n/a	<1	2.5	<1	<1	n.d
Tx601 + FAW 8 hr	n/a	n/a	n.d.	1	<1	<1	n.d

n/a = sample not taken; n.d. = not detected; FAW = fall armyworm

Whole corn plants with and without fall armyworm larvae were exposed to SPME

fibers overnight and then manually injected into the injection port of the gas

chromatogram. In the presence of fall armyworm larvae, caryophyllene was detected in

Mp708 but not in Tx601. In the absence of fall armyworm larvae, caryophyllene was

detected in Mp708, but was not positively identified in Tx601. In addition, an infestation

of maize grown in the greenhouse with wild fall armyworm larvae was investigated;

whorl tissue from infested plants was excised and volatiles collected. Caryophyllene was

detected at 200 kcounts in Mp708 but only 25 kCounts in Tx601 tissue. This data

follows the pattern of large amounts of caryophyllene detected in Mp708 but not in

Tx601, and provides evidence that excess caryophyllene production by Mp708 can also

occur in a natural setting with wild fall armyworms.

Caryophyllene Preference Study

  Results of the caryophyllene preference study indicate that the fall armyworm larvae are not repelled by pure caryophyllene. The location of the fall armyworm larvae after 8, 24, and 48 hours was random; half of the larvae were located on the Tx601 tissue with caryophyllene, and the other half were feeding on the tissue with hexane.

Quantitative Real-Time PCR (qRT-PCR)

  Due to technical difficulties, the results of the qRT-PCR were inconclusive, and the procedure is currently undergoing troubleshooting. Negative controls displayed peaks, suggesting the possibility of contamination of plates or the work surface. Maize ubiquitin primers previously used for RT-PCR did not amplify the ubiquitin consistently, signifying that the quality of the cDNA template was not sufficient for analysis. The preliminary results indicated that TPS23 transcripts could not be detected in the range of the machine, again suggesting that the quality of the cDNA template was suspect. RT-PCR is currently being performed by collaborators in another laboratory, as well as our laboratory, to identify the source of the problem.
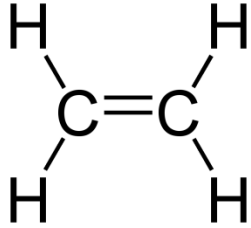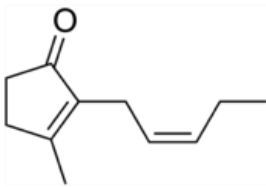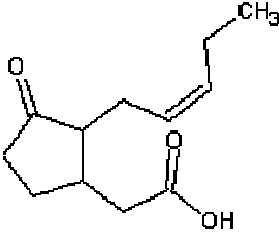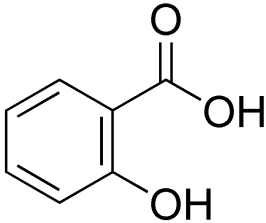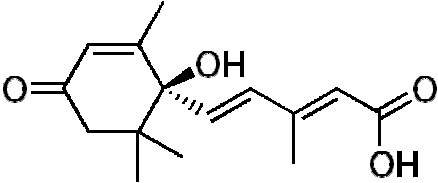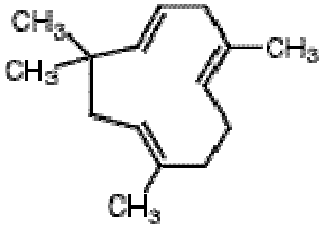
Discussion

  The results of the preference study suggest that caryophyllene is unlikely to play a role in repelling the fall armyworm larvae when it is released by Mp708. It is more probable that caryophyllene attracts natural enemies of the fall armyworm larvae, which is well established in published literature. It is also possible that caryophyllene might have an effect on the adult, moth stage of the fall armyworm's life cycle. Some volatile compound, or mixture of compounds, could affect whether or not a moth lays her egg mass on Mp708.

Analysis of caryophyllene levels over time in Mp708 and Tx601 in the presence and absence of fall armyworm larvae showed a consistent pattern. Caryophyllene was always detected in Mp708, whether or not there were fall armyworms present and regardless of the time point the sample was taken. Caryophyllene was rarely detected in Tx601 in the presence or absence of fall armyworms. While there was no pattern of caryophyllene production over time in Mp708, it is still apparent that caryophyllene is produced constitutively by Mp708.

APPENDIX A

CHEMICAL STRUCTURES OF COMMON PLANT VOLATILES

Table A.1   Chemical structures of common plant volatiles.

 Ethylene	 Jasmone
 Jasmonic Acid	 Salicylic Acid
 Abscisic Acid	 Caryophyllene