Tomato Plant Defenses against *Helicoverpa zea*

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ABSTRACT

When insects feed on plants, the oral secretions inevitably come in contact with wounded plant tissue and play very important role in regulating plant defense. These studies used tomato (Solanum lycopersicum) and Helicoverpa zea (H.zea) as model system to study how oral secretions affect tomato defense. In my first set of experiments, I found that saliva collected from the spinnerets of H. zea had higher glucose oxidase (GOX) activity, which induced tomato defenses. Plants that were wounded mechanically and then treated with with saliva or fungal GOX had higher levels of PIN2 gene expression in leaves and green fruits, while flowers and red fruit did not show comparable induction. GOX and saliva treatment also induce trichome number on newly growth leaf. Mechanically remove trichomes significantly increase insect damage and growth development.

Since trichomes contribute to plant resistance against herbivory by physical and chemical deterrents. To understand the role of different type trichome function in insect defense, the tomato trichome mutants were used to dissect trichome function in the defense. Study showed that hairless mutants had reduced number of twisted glandular trichomes (type I, IV, VI and VII) on leaf and stem, while woolly mutants had high density of non-glandular trichomes (type II, III and V) but only on the leaf. In both mutants, trichome densities were induced by methyl jasmonate (MeJA), but the types of trichomes present were not affected by MeJA treatment. Glandular trichomes contained high levels of monoterpenes and sesquiterpenes. High density of non-glandular trichome on leaves negatively influenced Leptinotarsa decemlineata (Colorado potato beetle, CPB) feeding behavior and growth, it stimulated H. zea growth. High glandular trichome density impaired H. zea growth, but had no effect on CPB. Quantitative real-time polymerase chain reaction (qRT-PCR) showed that glandular trichomes highly express protein
inhibitors (PIN2), polyphenol oxidase (PPOF) and hydroperoxide lyase (HPL) when compared to non-glandular trichomes. PIN2 in trichomes was highly induced by insect feeding in both mutant and wild type plants.

As defense signaling cascade is mediated by the synthesis, movement, and perception of jasmonate (JA) and its interaction with other plant hormones. In order to understand the interaction of ethylene and JA in regulating plant defense, the never ripe (Nr) mutant with a partial block of ethylene perception and the defenseless (defl) mutant with deficient in biosynthesis of JA were used to compare ethylene and JA interactions on tomato induced systemic defense. Proteinase inhibitor (PIN2) was used as marker gene to compare the plant response. Exogenous application of MeJA increased plants resistance to Helicoverpa zea and induced tomato defense gene expression and glandular trichome density on systemic leaves. Exogenous application of ethephon, an elicitor that releases ethylene, increased insect growth and interfere the tomato wounding response. Hormone assay showed ethephon treatment increase salicylic acid (SA) on the systemic leaves. These results showed that JA plays the main important role on systemic induced defense. Ethylene negatively regulated tomato systemic defense. This study also found that insect herbivory or MeJA treatment of the maternal generation induces transgenerational tomato defense in the offspring by increasing glandular trichome density and by priming defense genes that are regulated by plant phytohormone signals.
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List of abbreviations

- **woolly**: hairy mutant
- **hl**: hairless mutant
- **RU**: Rutgers wild type plants of woolly mutant
- **AC**: Alisa Craig, wild type plants of hairless mutant
- **MeJA**: methyl jasmonate
- **PIN2**: protease inhibitor 2
- **HPL**: hydroperoxide lyase
- **SlCycB2**: B-Type cyclin
- **PPOF**: polyphenol oxidase
- **CPB**: Colorado potato beetle
- **Nr**: Never ripe mutant
- **def1**: Defenseless 1 mutant
- **RU**: Rutgers wild type plants of *Nr* mutant
- **CM**: Castlemart, wild type plants of *def1* mutant
- **ERF1**: Ethylene response factor
- **PR1**: Pathogenesis related gene
- **ET**: Ethylene
- **SA**: Salicylic acid
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Chapter I

Insect herbivore-induced defense in plants

Introduction

Higher plants are continuously challenged by numerous environmental factors such as pathogen attack or herbivory. To avoid attacks or diminish the injury they cause, plants frequently respond by different defense strategies to protect themselves (Green and Ryan 1972; Howe and Jander 2008; Stout et al. 1996; Wu and Baldwin 2010). Among the strategies, synthesis of secondary metabolites and defense proteins are powerful chemical weapons that influence herbivore nutrition and growth development (Arimura et al. 2005; Baldwin et al. 2001; Kessler and Baldwin 2001; Vandenborre et al. 2010). These metabolites include glucosinolates, alkaloids, phenolics, proteinase inhibitors (PIN), polyphenol oxidases (PPO), lipoxygenases (LOX), among others. Besides these defenses, plants also can release volatile organic compounds (VOCs) to attract the predators and parasitoids of the herbivores (Arimura et al. 2001; Dicke and Dijkman 2001; Heil and Kost 2006). Physical defenses such as trichomes are another mechanism of defense in plants to prevent or diminish damage by herbivores. The occurrence of different trichome types has been associated with specific resistance (Boege and Marquis 2005; Dalin et al. 2008; Levin 1973). Evidence has accumulated over the past few years to indicate that defenses are regulated by a network of plant hormones (Berger and Altmann 2000; Steppuhn and Baldwin 2008).

When herbivorous insects feed on plants, they not only physical damage on the plants but also produce insect derived cues inevitably come in contact with the wounded plant tissue (Felton and Tumlinson 2008). These chemicals, produced when insect feed or oviposit on plants, are Herbivore associated Molecular Patters (HAMPs) called effectors and act as elicitors
of induced defenses. Effectors are insect secreted chemicals that suppress defenses. The ability of plants to recognize insect cues and respond is a form of plant immunity. As sessile organisms plants also have evolved diverse strategies to continuously adjust their response to adapt a broad range of stresses by transferring the plant defense to next generations, which is called transgenerational plant immunity.

**Effectors and HAMPs**

A diversity of plant pathogens, including bacteria, fungi, oomycetes and nematodes, secrete molecules when invading plants. The broader definition in plant-pathogen interactions of effectors include many molecules such as microbial (pathogen) -associated molecular patterns (MAMPs or PAMPs) (Hogenhout et al. 2009). HAMPs are secreted by insect herbivore and exhibit similar activity as MAMPs in regulating plant defense. Over the years evidence has accumulated that insects produce a cocktail of chemicals that modulate plant defenses (Felton and Tumlinson 2008; Mattiacci et al. 1995; Musser 2005; Schmelz et al. 2003b). Until now only a small quantity of herbivore-derived chemicals have been isolated and structure identified (Figure. 1).

a) Volicitin

\[
\text{N-(17-hydroxylinolenoyl)-L-glutamine} \quad \text{N-linolenoyl-L-glutamine}
\]

b) Caeliferin

CaeliferinA 16:1
Fig. 1 Structure of the known herbivore derived elicitors a) Fatty acid-amino acid conjugates, volicitin and N-Linoleoyl-glutamic acid. b) Caeliferins A and B isolated from *Schistocerca americana* c) Inceptin is the proteolytic product of plant chloroplastic ATP synthase γ-subunit found from *Spodoptera frugiperda* oral secretions d) Bruchins C isolated from pea weevil oviposition fluids e) Benzyl cyanide in *Pieris brassicae* oviposition fluids

**Fatty acid amino acid conjugates (FACs)**
Fatty acid amino acid conjugates (FACs) (Figure 1a) are the best studied HAMPs in plant-insect interactions. The FACs is composed of two moieties including fatty acid (or linolenic acid LA) and amino acid moiety (Glu or Gln). The fatty acid and amino acid originate from plants and insects, respectively and are synthesized in the insect midgut by the addition of a hydroxyl group and glutamine to linolenic acid obtained directly from the host plants. Volicitin (N-17-hydroxylinolenoyl-L-glutamine) was first isolated from beet armyworm (Spodoptera exigua) regurgitant and induce corn seedling to release volatile compounds, which attract parasitic wasps (Alborn et al. 1997). Several other FACs has been isolated from lepidopteran species, crickets and fruit flies (Halitschke et al. 2001; Pohnert et al. 1999; Spiteller and Boland 2003; Yoshinaga et al. 2007). Volicitin is a key component in regulating tritrophic interactions among plants, insect herbivores, and natural enemies of the herbivores.

Inceptin

In addition to FACs, the other types of HAMPs have been discovered. Inceptin (Figure 1b) was isolated from oral secretions of fall armyworm (Spodoptera frugiperda). It is a disulfide-bridged peptide (+ICDINGVCVDA-) and produced by proteolytic degradation of plant chloroplast ATP synthase γ-subunit. When fall armyworms attack cowpea plants, the ingested cATPC is cleaved in insect midgut and forms inceptins (Carroll et al. 2006; Schmelz et al. 2006). The inceptin could promote cowpea ethylene production and trigger increases in the defense related phytohormones salicylic acid and jasmonic acid.

Caeliferins
Caeliferins (Figure 1c) were isolated from the oral secretions of the grasshopper species *Schistocerca americana*. The caeliferins are composed of saturated and monounsaturated sulfated α-hydroxyl fatty acids with varies length from 15-20 carbons. The caeliferin A 16:1 is predominant and most active in inducing release of volatile compounds. While caeliferins A16:0, A17:0 and 18:0 are less active in inducing volatile compounds (Alborn et al. 2007).

**Bruchins and Benzyl cyanide**

Bruchins and benzyl cyanide are two substances found in oviposition fluids. Bruchins (Figure. 1d) were isolated from pea weevil (*Bruchis pisorum*) and elicit the tumor-like growth under the deposited egg, which inhibits the larvae from entering the pea pod. Benzyl cyanide (Figure. 1e) was isolated from butterfly (*Pieris brassicae*) which triggers the expression of defense-related genes (Hilker and Meiners 2002; Mumm et al. 2003). These chemicals regulate tritrophic interactions among plants, insect herbivores and natural enemies of the herbivores.

**Proteins**

In addition to the small molecules, the first reported HAMP protein is β-glucosidase, which is present in the regurgitant of *Pieris brassicae* caterpillar, triggered the emissions of volatile in cabbage plants (Mattiacci et al. 1995). Recently, glucose oxidase (GOX) was identified in the saliva of *Helicoverpa zea* (*H. zea*) oral secretion and mediates plant defense response. GOX converts glucose into gluconic acid and H₂O₂ and is the most abundant protein found in labial salivary glands of *H. zea*. Applications of GOX or labial gland extract suppress the wound-inducible production of foliar nicotine and volatiles in tobacco leaves. The bioassays
showed caterpillar survival and growth increased when fed GOX-treated leaves (Bede et al. 2006; Delphia et al. 2006; Musser et al. 2002).

\[
\begin{align*}
\text{Glucose oxidase} \\
\beta\text{-D-glucose + O}_2 & \rightarrow \text{D-gluconic acid + H}_2\text{O}_2
\end{align*}
\]

Functional genomics approaches haves also been utilized to identify the green peach aphid effectors (Bos et al. 2010). While feeding, aphid saliva secreted into plant cells and phloem. The saliva contains proteins such as Mp10, Mp42 and MpC002 with diverse activities and function as induce or suppress plant defense response. Also another recent study with green peach aphid – Arabidopsis model system indicates that salivary protein fractions between 3–10 kD acts as the potential salivary components that are recognized by the host plant to modulate plant defense responses (de Vos et al. 2010). But the detail structure and function in plant defense need more study to confirm. The interactions between plants and insects are complex and dynamic.

**Plant perception of insect herbivore**

The insect signal perception is the first step of induced plant defense and has been well investigated in many insects (Alborn et al. 1997; Alborn et al. 2000; Musser et al. 2002; Voelckel and Baldwin 2004). Because of different feeding behaviors, the insects use different methods to deliver their effector to the plants and the plants may have different perception mechanism. Phloem-feeding insects, such as aphid and whiteflies, could directly deposit their signals into plant phloem, which alter plant defense signaling and plant development. The insects use stylets to navigate the cuticle, epidermis, mesophyll and established feeding sites in phloem sieve elements (SEs) on host plants (Will and van Bel 2006). During the stylet penetration in
plant tissue and feeding from the phloem sap, they inject salivary secretions into the plant. More studies suggested the phloem-feeding insects use similar strategies as pathogens to introduce effectors into plant cells by many biochemical and cellular process (Bos et al. 2010). When the stylets pierce into phloem SEs, plants repair the SEs wounds by depositing callose and proteins to rapidly seal plasma membranes to prevent the leakage of phloem sap into apoplast (Walling 2009). As a consequence, plant perception of the effectors activates plant signaling pathways to regulate large set of genes that shape the appropriate defense responses.

The Hessian fly (Mayetiola destructor HF) has extremely minute mandibles which are not well structured for chewing, produce a shallow punctures on the leaf surface and release salivary secretions. This process rapid changes the surface wax composition, increases cell permeability and results in the diffusion of nutrients to the leaf surface during early stages of the interaction (Chen et al. 2004a). The resulting gene-for-gene recognition of larval salivary components initiates chemical and physical defenses. Previous studies showed that the RXLR protein motif mediates oomycete and fungal effectors entry into the plant host cell through the plasma membrane. Similarly the most recent study on HF study showed the Ave gene in HF genome encodes the effector proteins which have a modular structure contain RXLR-like motif and the structure resembles the effectors of filamentous plant pathogens alter plant immunity (Chen et al. 2010).

Chewing insects release oral secretions during feeding and plants perceive insect attack through the direct detection of these secretions. For example, maize (Zea mays) and tobacco (Nicotiana attenuata) can directly perceive the fatty acid amino acid conjugates (FAC) and induce volatile emissions. Radiolabeled volicitin was identified in the caterpillar regurgitant and recovered from corn leaves after feeding by Spodoptera exigua (Truitt et al. 2004).
saliva of Lepidoptera is released from the labial glands via the spinneret and one of the key functions is to facilitate feeding and digestion (Eichenseer et al. 1999; Felton and Eichenseer 1999). The immune-labeling method was used to confirm \textit{H. zea} release saliva during feeding. The results showed that larvae secret microgram amounts of saliva on the leaf surface during a few hours feeding (Peiffer and Felton 2005).

\textbf{R-gene mediated plant resistance}

Given the diversity of insect herbivores, the inducible defenses may respond differently in the modulating plant insect interactions. The induced defense response to phloem-feeding insects is similar to the plant defense to pathogens (Bhattarai et al. 2007; Walling 2000). Plants recognize pathogens through the pattern recognition receptors (PRRs) located on the cell surface and result in PAMP (MAMP) triggered immunity (PTI). To counter PTI, the pathogens develop mechanisms to secrete and deliver effectors into host cells which in turn triggers plant disease resistance (R) protein production (Dangl and Jones 2001). Previous study in a few systems showed that R gene products mediate resistance to phloem-feeding insect herbivore. The R gene in plants encodes NBS-LRR (nucleotide binding site-leucine rich repeat) proteins which recognize the insect hemipteran herbivore. \textit{Mi-1} gene in tomato encodes a protein sharing structural features with the NBS-LRR confers resistance to potato aphid, white flies and nematodes (Casteel et al. 2006). Another NBS-LRR protein, encodes by melon \textit{Vat} gene, confers increased resistance to \textit{Aphis gossypii} (cotton aphid) and the transmission of plant virus by this aphid species. The most recent finding showed that Bph14 gene encodes a coiled-coil NBS-LRR which resistance to rice brown plant hopper \textit{Nilaparvata lugens} (Chen et al. 2004b; Wang et al.)
These studies showed that some insect and plant interactions may fit the gene for gene model (Dogimont C et al. 2010).

**Induced plant defense**

Plants use constitutive and/or induced defenses against insect herbivory. One group of defenses are secondary metabolites; glucosinolate, phenolics, tannins and alkaloids are some of the most studied toxic secondary metabolites that play important roles in defense against herbivory (Howe and Jander 2008). The other group is anti-digestive or anti-nutritive proteins. In addition to direct defenses induced by insect feeding, plants also produce volatile organic compounds (VOCs) in response the damage. VOCs released by herbivore-attacked plants may attract arthropod predators and parasitoids in plants. One study showed that volicitin (17-hydroxylinolenoyl-L-Gln) in the regurgitant of *Spodoptera exigua* (beet armyworm caterpillars) activates the emission of VOCs when applied to the damaged maize leaves (Truitt and Pare 2004). The VOCs are high specific and serve as attractants for female parasitic wasps, which parasitize herbivore larvae (De Moraes et al. 1998).

When herbivore effectors or HAMPs are released on the plants, it could trigger a series of molecular events in plant cells. Ca$^{2+}$ has been recognized as secondary messenger involved in plant signaling. Under normal condition, the cytosolic Ca$^{2+}$ content are lower than in organelles. Trans-membrane ion fluxes were generated when insect effectors release at wounding site. The changes in intracellular Ca$^{2+}$ further activate calmodulins, calmodulin binding proteins, calcium-dependent protein kinase (CDPKs) and other calcium-sensing proteins that promote downstream signaling to modulate plant defense response (Maffei et al. 2006; Maffei et al. 2007).
The mitogen-activated protein kinase (MAPK) signaling pathway plays a central role in mediating plant response to herbivore attack. MAPKs are phosphorylated by MAPK kinases (MAPKKs) at the threonine and tyrosine residues located in the activation loop (T-loop) between subdomains VII and VIII of the kinase catalytic domain. Several MAPKs involved in plant defense have been identified (Heinrich et al. 2011). *Nicotiana attenuata*, when attacked by *Manduca sexta* (*M. sexta*), recognizes the FACs in *M. sexta* oral secretions (OS) that are introduced into wounds during feeding and rapidly activates two MAPKs, salicylic acid-induced kinase (NaSIPK) and wound-induced protein kinase (NaWIPK) which are required for the herbivory-induced biosynthesis of jasmonic acid (JA) and ethylene (Wu et al. 2007). Virus induced gene silencing (VIGS) reduce MAPK expression confirming their important role in plant defense. In Arabidopsis, activated (phosphorylated) MAPKs can directly phosphorylate certain downstream targets and stimulate induced defense (Beck et al. 2011; Chang and Karin 2001).

Hormonal signals involving jasmonate acid (JA), salicylic acid (SA) and ethylene (ETH), constitute a complex signaling network leading to defense-related gene activation and regulation (Chini et al. 2007; Howe and Jander 2008; Ludwig et al. 2005; O'Donnell et al. 2003). JA is a naturally occurring, non-toxic compound and a member of plant hormones which plays an important role in plant development and control plant defense against herbivores (Wasternack et al. 2006). The synthesis of JA is occurs in chloroplasts and peroxisomes through the octadecanoid pathway. In chloroplasts, the reaction begins with linolenic acid (LA 18:2and 18:3), it is catalyzed by lipoxygenases (LOXs) to form the 13-hydroperoxide of linolenic acid. The 13-hydroperoxide further processed by allene oxide synthase (AOS) and allene oxide cyclase (AOC) to form 12-oxophytodienoic acid (OPDA) (Bonaventure et al. 2011; Wasternack et al. 2006).
2006; Weber et al. 2004). After transport to peroxisomes, ODPA reductase (OPR3) catalyzes the OPDA, followed by three cycles of β-oxidation to form JA by shortening of the hexanoic and octanoic acid side chains (Figure 2) (Wasternack et al. 2006; Wu and Baldwin 2010). It is well established that JA levels increase in response to wounding, herbivory, elicitor treatment or other abiotic stress (Constabel et al. 1995). DNA microarray studies showed that the jasmonate pathway has a dominate role in regulating global changes in gene expression in response to both mechanical wounding and herbivory (De Vos et al. 2005).

Insect feeding triggers the expression of plant protease inhibitors (PIs) and other defense compounds that are regulated by the JA-pathway (Creelman and Mullet 1995; Ryan 1974). These compounds inhibit digestion in the larval midgut and affect insect growth development (Farmer and Ryan 1992; Pearce et al. 2001). Other enzymes such as polyphenol oxidase (PPO), lipoxygenase (LOX), arginase (ARG) and threonine deaminase (TD) may reduce the nutritional quality of plants.

SA is also a signal modulating plant defenses. SA is synthesized from chorismate through two reactions catalyzed by isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL). It’s well studied that SA play a central role as a signaling molecule involved in plant defense. SA regulates many pathogenesis-related (PR) genes which are antifungal hydrolases.
targeted to the plant cell wall (Traw and Bergelson 2003). One studied showed that an Arabidopsis transgenic line expressing the salicylate hydroxylase gene (NahG) had reduced the levels of SA and were more susceptible to *P. syringae* (Glombitza et al. 2004). A few reports implicate SA in defense against, but mostly focus on sucking insects like aphids and thrips. One mutant, the non-expressor of pathogenesis related genes1 (NPR1) showed enhanced resistance to aphids (Cole et al. 2004). Application of exogenous SA activates expression of plant pathogenesis-related (*PR*) genes and induces plant resistance to *H. zea* and triggers the accumulation of SA in cotton, tomato and tobacco (Bi et al. 1997).

Ethylene, as gas hormone, in addition to the central role in many plant physiological process throughout plant development including seed germination, cell elongation, flowering, fruit ripening, organ senescence could also elicits plant defense (Zhang et al. 2010). Ethylene is an important signal during herbivory, herbivore attack could enhance the ET burst (Kahl et al. 2000; Schmelz et al. 2003a). In many cases, ET has been shown to act as important modulator of plant response to SA and JA. Other wound signals include abscisic acid (ABA), reactive oxygen species (ROS) and nitric oxide (NO) (Adie et al. 2007; Klessig et al. 2000; Liu et al. 2010).

**Cross talk of hormone in plant defense**

Hormone cross talk is a process in which different hormone signaling pathways act antagonistically or synergistically to regulate plant development and/or adaptation environment stresses (Verhage et al. 2010). There is ample evidence for cross talk between SA, JA and ET signaling pathways in plant immunity (Pieterse and Dicke 2007).

**SA-JA** The interaction between SA and JA signaling appears to be complex and there is evidence in both positive and negative interaction. JA may collaborate with the SA response.
pathway to trigger an indirect defense against an insect herbivore (Van der Ent et al. 2009). Walling (2000) found that phloem-feeding insects may induce both SA and JA dependent pathways. Simultaneous expression of SA and JA pathways is also observed in Mi-resistance gene mediated defense in tomato to aphid feeding (de Vos et al. 2010). There is increasing evidence that JA and SA are involved in negative cross talk in plant defense networks. For instance, herbivorous nymphs of the silver-leaf whitefly could activate the SA signaling pathway but suppress the JA-dependent defense signaling. Some reports indicate that synergistic interaction between JA and SA. SA could repress JA mediated genes such as PIs and PPO (Doares et al. 1995; Thaler 2002). Felton et al. (1999) observed an inverse relationship between endogenous concentrations of SA and JA in tobacco. The JA mutants, mitogen-activated protein kinase4 (mpk4), suppress the SA insensitivity2 (ssi2) and coi1 (Chini et al. 2009; Kachroo et al. 2005; Liechti et al. 2006). These studies provided genetic evidence that JA signaling negatively regulated the expression of SA mediated defense in Arabidopsis (Gfeller et al. 2006; Liechti et al. 2006).

**SA-ET** Limited data shows both positive and negative interaction between SA and ET signaling pathway. The accumulation of SA in Xanthomonas campestris infected plants is dependent on ET synthesis, because no SA accumulation occurs in the ET-insensitive mutant. Microarray results showed SA and ET may coordinately induce defense-related gene expression (Odonnell et al. 1996). However the SA-dependent expression of PR genes didn’t require ET signaling.

**JA-ET** There are two modes of interaction between JA and ET. One is synergistic and the other is antagonistic (Pieterse and Dicke 2007). Several studies provide evidence for the positive interactions. For example, both JA and ET signaling are required for the expression of
the defense related gene PDF2 in response to the infection of *A. brassicicola* in Arabidopsis (McConn et al. 1997). Treating plants with ethephon, a synthetic ET donor, could elevate the JA, while blocking the ET with 1-MCP could reduce herbivory-induced volatile emission which are regulated by JA (Schmelz et al. 2003c). In tomato, the JA and ET interaction is responsible for the induction of proteinase inhibitors (PIN2) gene after wounding. Although the ET alone is not able to induce the PIN expression, it cooperates with JA to synergistically induce the gene expression (O’donnell et al. 1996). Previous studied showed that ET antagonistically to suppress JA induced gene expression in nicotine biosynthesis and in wounded leaves of Arabidopsis (Shoji et al. 2008).

The effect of herbivory on plants involves a complex interplay among varied signaling networks. Increasing evidence suggests that different defense pathways are activated in plants response to different type of stress. A thorough knowledge at the molecular level of how cross talk occurs between different hormone pathways is necessary to understand how plants respond to different stresses and in identifying new ways improve plant defense responses.

**Trichome as first line of plant defense**

Trichomes are uni- or multicellular hair-like structures that develop from cells of the aerial epidermis and are produced by many plant species. Numerous studies have shown that leaf trichomes can serve multiple functions as structural and chemical against herbivores (Amme et al. 2005; Kang et al. 2010a; Levin 1973). In addition resistance to insect herbivory, trichomes also provides resistance to abiotic stress, such as drought or UV radiation.

The morphology and density of leaf trichomes vary among plant species. Arabidopsis foliar trichomes are unicellular, stellate, non-glandular trichomes that usually contain three
branches and are an excellent model to study all aspects of cell differentiation (Marks et al. 2009). The trichomes of tomato *Lycopersicon* (or now *Solanum lycopersicum*) were first described by Luckwill (Luckwill 1943) and categorized as seven types trichomes. The type I, IV, VI and VII are glandular trichomes which have a secretory head and an important site for biosynthesis and accumulation of a wide range of plant natural products. The type I trichomes have multicellular base and long multicellular stalk, while type IV trichomes have a unicellular base and short multicellular stalk compared to type I trichomes. The type VI trichomes containing the four-celled glandular head have been the most heavily studied trichomes in tomato. The type VII trichomes are irregularly shape and have short unicellular stalk (Luckwill 1943). The types II, III and V are non-glandular trichomes. The type II and III are similar with the multicellular and unicellular base individually, while the type V trichomes are short with unicellular base (Kang et al. 2010b; Levin 1973).

Trichome density is an important factor of resistance against herbivorous insects (Levin 1973; Traw and Dawson 2002a). The presence and density of leaf trichomes can influence both host-plant selection behavior and performance (i.e. growth, survival and fecundity) of herbivorous insects, but can be considered a relatively soft “weapon” in plant defense compared with many other traits that are lethal to insects. The role of glandular trichomes in resistance of *L. hirsutum* has been thoroughly explored by Gurr & McGrath (2002). Research has shown glandular trichomes of tomato species spp. are responsible for resistance to various insects (Kang et al. 2010a; Kang et al. 2010b). Studies showed the trichome density is negatively related to insect growth and experimental removal of leaf trichome results increasing insect performance on several species plants (Ashouri et al. 2001). Experimentally removing glandular trichome heads and exudates significantly decreased the mortality of neonates on the two accessions with
highest levels of resistance in their natural state. This supports similar findings from previous research conducted on the potato aphid (Gentile et al. 1969), H. zea (Dimock et al. 1982), L. decemlineata (Kennedy 2003) and T. vaporariorum (Gentile et al. 1969). Glandular trichomes can be viewed as a combination of a structural and chemical defense (Sarria et al. 2010). Non-glandular trichome had a negative effect on oviposition, hatching rate and survival of insects (Baur et al. 1991).

Some plant species respond to insect feeding or mechanical wounding by producing more trichomes on new growth leaves (Agrawal 1999; Dalin and Björkman 2003). The trichome density is typically increased between 25%-100% within days or weeks after the wounding (Baur et al. 1991). For example Björkman study showed that the trichome production was induced by leaf beetle herbivory in the willow Salix cinerea. The damage on the leaf could also change the relative proportions of glandular and non-glandular trichomes. Recent study on Arabidopsis trichomes showed jasmonate acid regulates the systemic trichome development (Karban et al. 2003; Traw and Dawson 2002). Application of jasmonic acid (JA) or methyl jasmonate (MeJA) and gibberellin (GA) increase trichome production on newly growth leaves but salicylic acid (SA) reduces trichome production. Thus the hormone levels in plants regulate trichome production on leaves (Boughton et al. 2005; Traw and Bergelson 2003). The abiotic stress such as drought and UV-radiation also could induce trichome production (Nagata et al. 1999).

The most remarkable feature of tomato trichomes is their capacity to produce and secrete a wide variety of plant secondary compounds including terpenoids (Bos et al. 2010; Kang et al. 2010a; Schilmiller et al. 2010), phenolics (Gang et al. 2001), sucrose esters, methylketones (Ben-Israel et al. 2009) and organic acids (Voirin et al. 1993). Many of the trichome-borne compounds have significant value as fragrances, food additives or natural pesticide (Wagner
Due to the importance of trichome chemicals in defense, more studies need to focus on systemic analysis of the metabolites in trichomes and elucidation of their biosynthetic pathway (Schilmiller et al., 2008, 2009, 2010; Kang, 2010). For example, type VI trichomes in tomato produce 2-tridecanone and other methylketones that are high toxic to some arthropod pests of tomato. In *Nicotiana species*, the accumulation of nicotine in trichomes induced by jasmonate is toxic to *Manduca sexta* (Preston et al. 2001).

In contrast to the broad knowledge on trichome morphology and chemistry, the protein complement of the trichome is relatively less studied. The proteomics and molecular techniques are useful tools to analyze the enzyme and pathway of the chemicals in trichomes. Shotgun proteomics analysis allows the identification and relative quantification of the proteins in trichomes. Extensive DNA sequencing was combined with proteomics aids in the genes and proteins expressed in trichomes. Studies showed that the monoterpene (MTS1) and sesquiterpene (SST1) synthesis genes play a key role in regulating terpenoid production in glandular trichomes in tomato (Besser et al. 2009). Proteinase inhibitor proteins, interfere the herbivory physiology by inhibiting the digestive process of insects, are constitutively expressed in trichomes. In addition to proteinase inhibitors, the trichomes of many *Solanum* species also accumulate polyphenol oxidase which plays an important role in defense insects and pathogens. Trichome-based host plant resistance may have the potential to reduce pesticide use during tomato production.

Because tomato (*Solanum lycopersicum*) is an economically important crop, it serves as a model for studying plant defenses against herbivores and diseases (Green and Ryan 1972; Howe and Jander 2008; Mirnezhad et al. 2010). Decades of research on tomato has shown that jasmonate signaling (JA) plays an important role in plant defense signaling against insect
herbivory (Bostock et al. 2001; Thaler et al. 2001). Trichomes in cultivated tomato and related wild species have been subjects of intense study for many decades (Steffens 1991; Kennedy et al. 1991; Peiffer et al. 2009).

In tomato, the effect of trichome-based resistance on insect herbivores has mostly focused on glandular trichomes and there has been considerably less study on the role of non-glandular trichomes against herbivores (Kang et al. 2010a). Because most cultivars possess both glandular and non-glandular trichomes, it is difficult to separate trichome function(s) in these commercial varieties. To study different trichome function in plant defense, the mutants with different type’s trichome will be good to study the function of trichome in plant defense.

**Mechanism of transgenerational plant defense**

Plants being have evolved diverse strategies to continuously adjust their response to a broad range of stresses and herbivore challenges. Herbivore resistance mediated by jasmonic acid (JA) and related metabolites within one generation has been extensively studied (Wasternack et al. 2006), but there is little investigation into the longer-term inducible defense in plants or how resistance may persist across generations.

Changes in the parental condition can affect the phenotype of their offspring. This process is considered as an epigenetic (non-genetic) phenomenon which can persist over many generations, since the parental effects significantly affect the phenotype of its offspring via transmission of non-genetic information. Agrawal and colleagues used wild radish (*Raphanus raphanistrum L. brassicacease*) and herbivory as model to examine the induced defense in the next generation, and they found when insects fed on maternal plants, the progeny were more resistant than progeny from undamaged plants (Agrawal 1999). In the field experiments, plants
exposed to herbivory in the early season had 60% higher relative fitness than undamaged controls (Agrawal 1999). Induced responses in wild radish showed variation corresponding with different insect herbivores. In some instances, the offspring of the damaged plants showed higher numbers of trichomes compared to the offspring from their undamaged parts (Agrawal 2001). A similar phenomenon was also observed that the increased glandular trichome density in the progeny of yellow monkey flower, *Mimulus guttatus*, when the parents were exposed to herbivory (Holeski 2007). Further the maternal generation exposed to herbivory also had positive effects on other traits in the offspring such as seed biomass and flower production (Streets and TL 2010). In addition, environmental factors can influence the properties of the offspring, when seeds from plants grown under warm temperature during flowering and seed development, the progeny showed higher nitrogen content, higher germination and growth rate, and increased seed biomass and seed production (BLÖDner et al. 2007). The mechanisms of transgenerational plant defense study could provide a useful tool for crop management (Chinnusamy and Zhu 2009).

Transgenerational plant defense means that the offspring’s immunity traits are acquired by the adult parent; in other words the adaptation of the maternal effects responding to the environment can improve the fitness and immunity traits of the offspring. Several studies have examined the contribution of maternal stress to offspring’s phenotype and fitness. Agrawal and colleagues used wild radish (*Raphanus raphanistrum L. brassicaceae*) and insect herbivory as model to examine induced defenses in next generation, and they found insect feeding on the maternal wild radish caused progeny to be more resistant than progeny from unfed wild radish (Agrawal 1999). Field experiments showed that plants exposed to herbivory in the early season had 60% higher relative fitness than un-exposed controls (Agrawal 1999). In some instances, the
offspring of the damaged plants showed higher numbers of trichomes compared to the offspring from their undamaged parents (Agrawal 2001). A similar phenomenon was also observed in the progeny of yellow monkey flower, *Mimulus guttatus*, when the parents were exposed to herbivores (Holeski 2007). Transgenerational induction of high trichome density could confer a selective advantage, if offspring are likely to experience the same herbivore pressure as their parents.

How plants transmit the stress responsive properties to the next generation, and the underlying mechanisms of transgenerational adaptation are currently not well known. The transgenerational defense should not result from random mutagenesis followed by the selection of the fittest individuals, as the frequency of spontaneous mutations is rather low. The genome stability is maintained by various repair mechanisms, and homologous recombination may serve as an important mechanism involved in repair (Schuermann et al. 2005). One recent study showed that the progeny of *Nicotiana tabacum* infected with tobacco mosaic virus exhibited a high frequency of rearrangements at disease resistance gene-like loci (Kathiria et al. 2010), and other couple studies also found the correlation between transgenerational transmission with the homologous recombination frequency (HRF) response to stress. These data suggest that continuous exposure to the stress may lead the evolutionary selection of the adaptive traits which are beneficial to the plants.

Among the inheritable genome changes, reversible DNA methylation and chromatin modifications are involved in the regulation of gene expression (Zilberman and Henikoff 2005). This reversible epigenetic adaptation could serve plants as an alternative strategy to environmental stresses. DNA methylation include both asymmetric (\(^5\)CpHpH)-methylation and symmetric (\(^6\)CpG and \(^6\)CpHpG)-methylation. The low level of DNA methylation frequently
correlates with the expression of stress specific gene; in one study, virus-infected tomato plants showed changes in DNA methylation at several marker loci (Mason et al. 2008). Another study showed that dandelions expose to SA led to genome wide and possible stress specific changes in DNA methylation and were transmitted to the immediate progeny (Verhoeven et al. 2010). DNA cytosine methylation in gene promoter is associated with repressive chromatin and with repression of gene. In maize roots, cold stress induced expression of ZmMII was correlated with the reduction of methylation in the DNA of nucleosome core (Steward et al. 2002). In Arabidopsis, the drought induced expression of stress responsive gene is associated with an increase in H3K4 trimethylation and H3k9 acetylation. Histone variant H1 in tomato was shown involved in regulation of stomatal conductance from drought afflicts (Tsuji et al. 2006).

Another epigenetic factor, short interfering RNAs (siRNAs), is also active in the stress response, the siRNAs are able to move between cells and through the plant vasculature, and play import role in signal transduction during the plant development (Chitwood and Timmermans 2010). In fact, a large number of miRNAs and other small regulatory RNAs are encoded by the Arabidopsis genome and that some of them may play important roles in plant responses to environmental stresses as well as in development and genome maintenance (Sunkar and Zhu 2004). Endogenous siRNAs that are regulated by abiotic stress have been identified in Arabidopsis (Sunkar and Zhu 2004). These findings suggest that biotic and abiotic stress could shape the plant genome by different mechanisms, the heritable epigenetic modification may provide within generation and transgenerational stress memory.

It would be highly interesting to find distinct profile of DNA or histone methylation, siRNA of specific stress response, which will supply more understanding of transgenerational defense and possible as guidance for crop management and insect-resistant improvement.
Perspectives and future directions

Many research studies have contributed significant progress to deciphering the molecular mechanism of plant immunity, including the roles of herbivore effectors in perception and defense. But until now only very few number of HAMPs and effectors have been identified. Recent reports from *Arabidopsis thaliana* have demonstrated how pathogens may exploit protein interactions to manipulate a plant’s cellular machinery. More study is needed to focus on insect derived effectors and their function in plant-insect interactions. It may be that insect herbivores produce cocktails of effectors as seen with pathogens, and that these cocktails broadly induce host-plant defenses. The study of effector networks will contribute to understanding plant-insect interactions and may offer clues to improve plant production. The protein-protein interaction map would be a good technique to study the complex signaling networks of plant and insects.

Main points

- When herbivorous insects feed on plants, they cause not only physical damage, but also produce chemicals which may be perceived by the plants and thus activate plant defenses.
- Plants use combinations of constitutive and inducible defense traits to defend against insect damage. The defense response is regulated by several hormone signaling networks involving jasmonic acid, salicylic acid, and ethylene.
- Induced plant defenses may even persist across generations. Such transgenerational resistance may provide a novel approach to improve crop production with fewer environmental problems than current pest management approaches.

The model system in this study
Because of its economic importance, relatively small genome, short life cycle, and ease of growth and maintenance, tomato *Solanum lycopersicum* (formerly *Lycopersicon esculentum*) has long served as a model system for plant genetics, development, pathology, and physiology. Decades of applied and basic research has focused on defenses employed against tomato diseases and insect herbivores, including caterpillars (Lepidoptera) and beetles (Coleoptera), both groups of which include diverse and important group of agricultural insect pests that in addition to damaging tomatoes cause widespread economic damage on food and fiber crop plant species, fruit trees, forests, and stored grains.

In this research the generalist noctuid *Helicoverpa zea* and Solanaceae specialist, Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*) were used as the main insects to study tomato-insect interaction. The larvae of *H. zea* (sometimes called the tomato fruitworm) are a major agricultural pest because they feed on many different plants during the larval stage. Management of the fruitworm requires careful monitoring for eggs and small larvae. It’s essential to control the pest before the larvae enter fruit, where they are protected from insecticide sprays and predators. Colorado beetle is a serious pest of potatoes and also cause significant damage to tomatoes and eggplants. Both larvae and adults feed on the plant leaves can cause damage.

**Objective 1: Determine the function of *H. zea* saliva in tomato defense**

Since tomato fruit is the target for food production in the field, it is imperative to better understand the response of fruit to herbivores. Because of small size, short life and small genome (950 Mb), the micro-tom tomato was used in the studying of *H. zea* saliva function (Yano et al.
The main hypothesis is that *H. zea* saliva differentially modulates plant response at different plant growth stages.

Previous study showed the glucose oxidase (GOX) is the primary salivary protein from the caterpillar *H. zea* ((Musser et al. 2005). In this study I compared the plant defense response to *H. zea* saliva at different growth stages including defense gene expression and trichome induction.

**Objective 2: Compare the function of different types of trichomes in defense**

Seven types of trichome had been described in tomato, most cultivars possess both glandular and non-glandular trichomes, it is difficult to separate trichome in the commercial varieties. Different trichome mutants were used to compare trichomes role in tomato defense. In this current study, I used two mutants with differing trichome phenotypes to examine the role of glandular and non-glandular trichomes in resistance to the solanaceous specialist *L. decemlineata* and the generalist *H. zea*. I hypothesized that both glandular and non-glandular trichome phenotypes will provide different contributions to defense against these herbivorous insect species.

**Objective 3: Cross talk of JA and ET in tomato defense**

Tomato has been a primary model for elucidating the signaling pathways and induced defenses against herbivores. The effects of herbivore on plants involve a considerably more complex interplay among varied signaling networks than earlier thought. Many researchers have found that herbivores could elicit multiple defense pathways, which may result in cross talk among the pathways (Felton and Korth, 2000). Most of the research has focused on signaling
and rapidly-induced defenses in leaves (Jose, 2001). The main objective is to study the interaction of JA and ET on systemic induced defense.

**Objective 4: Transgenerational defense in tomato**

Previous study showed that changes in the parental condition could affect the phenotype of their offspring. For example, plants exposed to herbivory in the early season had 60% higher relative fitness than undamaged controls (Agrawal 1999). Induced responses in wild radish showed variation corresponding with different insect herbivores. In order to test whether tomato plants have transgenerational defense insect feeding, I exposed micro-tom tomato plants to *H. zea* and collected seeds to compare how the progeny of treated plants respond to insect feeding.
References


Agrawal AA (1999) Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80: 1713-1723


Ashouri A, Michaud D, Cloutier C (2001) Unexpected effects of different potato resistance factors to the Colorado potato beetle (Coleoptera : Chrysomelidae) on the potato aphid (Homoptera : Aphididae). Environ. Entomol. 30: 524-532


Baur R, Binder S, Benz G (1991) Nonglandular leaf trichomes as short-term inducible defense of the grey alder, &lt;i&gt;Alnus incana&lt;/i&gt; (L.), against the chrysomelid beetle, L. Oecologia 87: 219-226


Bhattarai KK, Li Q, Liu Y, Dinesh-Kumar SP, Kaloshian I (2007) The Mi-1-Mediated Pest
Resistance Requires Hsp90 and Sgt1. Plant Physiol. 144: 312-323
plant resistance to herbivory. In: Schaller A (ed) Induced Plant Resistance to Herbivory, pp 89-105


Shoji T, Ogawa T, Hashimoto T (2008) Jasmonate-Induced Nicotine Formation in Tobacco is Mediated by Tobacco COI1 and JAZ Genes. Plant Cell Physiol. 49: 1003-1012


Truitt CL, Pare PW (2004) In situ translocation of volicitin by beet armyworm larvae to maize and systemic immobility of the herbivore elicitor in planta. Planta 218: 999-1007


Chapter II

Salivary glucose oxidase from caterpillars mediates the induction of rapid and delayed-induced defenses in the tomato plant

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Abstract

When *Helicoverpa zea* (*H. zea*) feeds on plants, the oral secretions inevitably comes in contact with wounded plant tissue and play very important role in regulating plant defense. The proteomic analysis showed glucose oxidase (GOX) is the main protein in *H. zea* saliva. Saliva collected from spinneret had higher GOX activity, while the regurgitant showed no GOX activity. Mechanical ablated spinneret could stop saliva release which is confirmed by immunodetection method with anti-GOX. Intact insect fed significantly induce proteinase inhibitor (*PIN2*) gene expression and trichome number on the newly growth leaves compared to the ablated fed. Mechanical wounded with saliva or fungal GOX treatment at different growth stages, showed different induction. Both GOX and saliva induce leaf and green fruit *PIN2* gene expression, while the flower and red fruit didn’t show any induction compare to non-treatment. GOX and saliva treatment also induce the newly growth leaf trichome number. Experimentally remove trichome significantly increase the leaf damage and insect growth. These findings showed that *H. zea* saliva play important role in tomato defense, including induces defense gene expression and increase trichome density on newly growth leaves which affect insect growth.

Key words: *Helicoverpa zea*, Saliva, Glucose oxidase, *Solanum lycopersicum*, Induced defense, trichome
Introduction

Emerging evidence indicates that when herbivores initiate feeding on a host plant, they not only mechanically damage plants, but also present “cues” that the plant perceives and uses to rapidly mobilize induced defenses in response to attack (Fatouros et al. 2008; Maffei et al. 2006; Mithofer et al. 2005; Musser et al. 2002; Peiffer et al. 2009; Tooker et al. 2010). Over the years evidence has accumulated that insect derived a cocktail of chemicals during feeding which modulate plant defenses (Felton and Tumlinson 2008; Mattiacci et al. 1995; Musser 2005; Schmelz et al. 2007). These include fatty acid-amino acid conjugates (FACs) identified in various lepidopteran larvae (Alborn et al. 1997; Spiteller et al. 2000), β-glucosidase from larvae of white cabbage butterfly (Pieris brassicae)(Mattiacci et al. 1995), inceptins isolated from fall armyworm (Spodoptera frugiperda) larvae and caeliferins identified from grasshopper species (Schistocerca americana) (Alborn et al. 2007). Glucose oxidase (GOX) was first characterized a salivary enzyme from Helicoverpa zea (H. zea) (Musser et al. 2002). Although already identified some chemicals from specific species of insects, our understanding of the chemistry of these secretions is still very much in its infancy. Identification more chemicals in the oral secretions will be better to study the oral secretions function.

To investigate oral secretion specific function, researchers have applied larval oral secretions or regurgitant to mechanical wounds or directly mechanically stop secretions. Several studies demonstrated that application of insect oral secretion to artificial wounds can mimic most plant responses to herbivory (Maffei et al. 2004; Musser et al. 2006). For example, β-glucosidase from Pieris brassicae induces the release of a volatile blend when applied to wounded cabbage leaves, causing the attraction of the parasitoid Cotesia glomerata (Mattiacci et al. 1995). Fatty acid–amino acid conjugates (FACs) are produced by many lepidopteran caterpillars and induce
direct and indirect defense responses in plants (Alborn et al. 1997; Roda et al. 2004). In general, these insect oral secretions are important effectors which act as elicitor or suppressor in mediating of indirect or direct defense (Consales et al. 2011; Delphia et al. 2006; Felton and Eichenseer 1999; Musser et al. 2005; Weech et al. 2008a; Will et al. 2007).

Plants defensively respond to insect herbivory by both general and specific induced defense has been documented in a voluminous literature (Kaloshian and Walling 2005; Walling 2000). Among the strategies, the synthesis of secondary metabolites in plants is the powerful chemical weapon and function similar as toxins to inhibit herbivore's nutrition and growth (Bergey et al. 1999; Chao et al. 1999; Hui et al. 2003). Besides chemicals weapon plants also could attract the predators and parasitoids of herbivore through release of volatile organic compounds (VOCs) (Arimura et al. 2005). The more extensive research found that the plant induce defense were regulated by plant hormones (Berger and Altmann 2000; Steppuhn and Baldwin 2008), such as Jasmonic acid (JA) which is rapidly synthesize from fatty acid precursor following herbivore attack and play a key role in induction of plant defense to chewing herbivore. The cascade of proteins include proteins such as proteinase inhibitors (Diez-Diaz et al. 2004; Thipyapong et al. 2007); polyphenol oxidases (Constabel et al. 1995; Felton et al. 1989; Felton et al. 1992); ascorbate oxidase (Felton and Summers 1993); leucine aminopeptidase (Pautot et al. 1993); arginase and threonine deaminase (Lin et al. 2008) which are regulated by the octadecanoid pathway (Farmer 2007; Howe et al. 1996; Victoriano and Gregório 2002). In addition to these proteins, the role of glandular trichomes in resistance of insect feeding has recently been more thoroughly explored by Gurr & McGrath (2002). Glandular trichomes in tomato are a formidable defense against some herbivores by secreting sticky, noxious compounds that affect insect growth (Kang et al. 2010a; Kang et al. 2010b). The production of
these trichomes is induced by insect feeding or application of methyl jasmonate (Agrawal 1999; Boughton et al. 2005).

Every plant has different development growth stage during life. The production of defense response insect herbivore may be different. The induced plant defense has been mainly focused on studied in leaves since the biosynthesis of defense compounds is thought to be energy and nutrient demanding (Karban et al. 2000). Defense on fruits, despite being the preferred feeding site of a number of caterpillar pests such as *H. zea*, has been largely ignored. Only a few study investigate the induce defense in plant reproductive tissues (McCall and Karban 2006; Wang et al. 2009). Because tomato fruit is the main target for food production, it is imperative to better understand the response of fruits to herbivory.

Because of the complexity of plant signaling networks, there are multiple points at which caterpillar secretions may intercept or amplify signaling components. For example, the chewing herbivores use mandibles and maxillae for chewing and processing the food. By way of background, their secretions may arise from regurgitant derived from the digestive system (Peiffer and Felton 2009) or from saliva produced by the salivary glands (Felton 2008a). The previous study showed that glucose oxidase from the labial glands may suppress wound-induced accumulation of nicotine in tobacco (Musser et al. 2002). Regurgitant is known to contain scores of proteins (Liu et al. 2004), as well as fatty acid-glutamine conjugates such as volicitin, which elicit the production of plant volatiles—important components of indirect defense and plant-plant signaling (Engelberth et al. 2004; Mori et al. 2001). We initiated this investigation in tomato to examine the role of saliva in mediating tomato defense at different growth stages. This study provided evidence of saliva as effectors in induced plant defenses at different growth stages. The
micro-tom tomato is the best for us to study the defense at different growth stage because of its short generation time (ca. 55days), diminutive size and known JA signaling pathway.

Material and Methods

Plants and insects

The tomato (*Solanum lycopersicum*, cv. Better boy and Micro-Tom) were grown as described (Peiffer and Felton, 2005) and used at different growth stage. For all induction experiments, plants were maintained in greenhouse under 800W Super Spectrum lights (Sunlight Supply Co., Vancouver, WA). Tomato fruit worm (*H. zea*) eggs were purchased from BioServ (Frenchtown, NJ) and reared on a wheat germ and casein-based artificial diet (Bansal et al. 2011) with ingredients from Bioserv in the Entomology Department, Penn State University (University Park PA, USA).

Collection of *H. zea* saliva and regurgitant

To collect *H. zea* saliva, 5th instar larva were chilled on ice. Flaccid larva were then immobilized in a metal hair clip and observed with a dissecting microscope. As the larva returned to room temperature, the saliva was collected from spinneret secretions in glycerol with gel loading pipette tip (VWR, West Chester, PA) and pooled 10 caterpillar collection as one sample dispensing them into micro-centrifuge tube on ice. Regurgitant was collected from these larvae by gently squeezing the caterpillars, collecting the oral secretions using pipette and dispensing them into a micro-centrifuge tube on ice. All the collections were stored at –80º C until use.

Proteomics of *H. zea* Saliva
For proteomic identification of saliva proteins, saliva was collected as described above, except 5mM EDTA in 50 mM Tris-HCl, pH 8.0 was used in place of glycerol. Saliva was collected from 100 *H. zea* larva into 30 µl of buffer and stored at -80 C. NanoLC was carried out using a Dionex Ultimate 3000 (Milford, MA). Mobile phase solvents A and B were 0.1% TFA (v/v) in water and 0.1% TFA (v/v) in 80% Acetonitrile respectively. Tryptic peptides were fractionated at a flow rate of 0.6 µl/min using linear gradients and the following program: 5% B for 5min, 5 to 15% B over 5min, 15 to 60% B over 40 min, 60 to 95% B over 1 min and hold for 5 min. The mobile phase was ramped back to the initial conditions. Fractions were collected at 20-seconds intervals followed by Mass Spectrometry analysis on Applied Biosystems proteomics Analyzer. Peptides were searched against an insect database.

**Glucose oxidase (GOX) activity assay and visualize on leaf**

The GOX activity was determined according to the method described by Eichenseer et al. (1999). The glucose (0.1mM) and o-dianisidine (2.1M) were use as reaction cocktail with the sample and measure spectrophotometric at 460nm. The regurgitant was used directly after centrifuge. Because small volume of saliva, it was combined with 30µl PBS buffer for GOX activity assay. To compare whether ablated and insect fed affect GOX on leaf surface, tissue printing experiments were followed Peiffer and Felton description (2005). Ablated and intact six-instar larvae were fed overnight on detached tomato leaf. Then after transfer to nitrocellulose and superblock, and incubated with anti-GOX, detected with vector ABC Elite and Vector DAB kit.

**Insect feeding induce defense**

Fifth-instar *H. zea* larvae were ablated using previous described method (Peiffer, 2005). The caterpillars were chilled on ice until flaccid and then the spinneret was cauterized by toughing with a heat pen (Electron Microscopy Sciences, Fort Washington, PA). The ablated
larva was allowed to recover and feed on artificial diet for 7 hrs, then starved overnight ready for experiments. At 4\textsuperscript{th} node stage, the ablated and intact larvae were caged individually on the fourth leaf with 6 hour feeding and then removed caterpillar and cages, empty caged plants were use as control. After 24 hours and 48hrs caterpillar feeding, 100mg caged leaf was harvest in liquid nitrogen and stored at -80 ° C for later RNA extraction.

**Wounding induce defense on different tissues**

To investigate the saliva function in regulating tomato defense, the collected saliva was directly applied to the wounded site. Better boy and Micro-Tom seedlings were both treated to compare different varieties response saliva treatment. The regurgitant was applied on leaf to study its function on tomato. To test how saliva directly induce tomato defense on different growth stages. We wounded tomato on different tissue by punching two ¼ inch diameter hole along the mid-vein of the terminal leaflet of the 4\textsuperscript{th} leaf. Fruits were wounded by punching one hole at the side of the fruits. Green fruit is two weeks after flowering. Red fruit is at the beginning turning red. Flower treatment I wound at the pedicel. After wounding, we immediately pipette 20 µl of PBS buffer, \textit{H. zea} saliva or fungal GOX (20ng/µl) onto the edges of wounded site. Control plants were unwounded leaf, flower or fruit. After treatment 24 and 48 hours, 100mg plant tissues were harvest for RNA extraction and gene expression assay.

**Trichome induction and function**

To learn how \textit{H. zea} feeding and oral secretions affect glandular trichome induction, two methods were used to treat the plants. One treatment is ablated and intact \textit{H. zea} in caged plants feeding 6 hours then remove the cage. The other is direct wounding with saliva or fungal GOX treatment. Each treatment replicated on 10 plants. Tomato plants were maintained in the
greenhouse for two weeks after wounding and secretion application, two leaf discs beside the main vein were sampled and counted as previously described (Boughton et al. 2005).

To test the function of trichome, we mechanically removed trichome by gently touching and rubbing with hands along the mid-vein of the leaves. In petri-dish we let 3rd instar larvae feeding for 24 hours and measure the damage by scanning the leaf and analysis with Sigma Scan. And also in the small cup we use 1st instar larvae for 5 days bioassay to measure the larvae weight.

**Quantitative Real Time PCR**

Tissue (100 mg), harvested from the area around the wound, was homogenized in liquid nitrogen and total RNA extracted with RNeasy Plus Mini-kit (Qiagen, Valencia, CA). 1μg purified RNA was used with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) to create cDNA. Real-time PCR primers were designed using Primer Quest Software (Applied Biosystems) (Table 1). All reactions used Power SYBR Green PCR Master Mix and were run on a 7500 Fast Real-Time PCR System (Applied Biosystems).

The housekeeping gene ubiquitin (Rotenberg et al. 2006) was used to normalize C(T) values. Relative quantifications, with unwounded plants as the reference group, were then calculated using the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001). To validate this analysis method, primer efficiency was analyzed by comparing the normalized C(T) values of 5 serial dilutions of cDNA. The primers used were list in Table 1. For statistical analysis, relative expression values were analyzed by ANOVA with MiniTab (Minitab Inc., State College, PA) using Fisher’s separation of means (P<0.05).
Results

GOX activity determination and detection

On the average we can collect about 5 µl of regurgitant and about 0.5 nl of saliva from each caterpillar. Although the volume is small, regurgitant contains about 0.12 µg/µl protein in the collections, and 10 caterpillar saliva collections contain approximately 560 ng proteins, as measured by a modified Bradford assay (Vincent and Nadeau, 1983). GOX activity assay showed regurgitant was no GOX activity, while saliva had high activity around 1.8 mol/min/mg proteins (Table 1).

To verify the ablation procedure successfully prevented release of saliva, we performed a tissue blot of leaves fed on by the treated caterpillars (Peiffer and Felton, 2005). Anti-GOX was used to detect GOX on leaf after *H. zea* overnight fed, there was significantly different between the ablated and intact fed on leaf. Most of the GOX were spread on the leaf with intact fed, while no GOX were detected on the leaf after ablated caterpillar fed. This confirmed that saliva was released from the caterpillar spinneret at the same time when insect feeding.

The collected saliva was sent to company for shotgun proteomic analysis of secreted salivary proteins from *H. zea* to identify potential candidates for plant defense elicitation. The number of mass spectral counts obtained for each protein provides a quantitative measure of protein abundance (Old et al. 2005). Of the 33 proteins that were identified, glucose oxidase (GOX) was by far the most abundant protein accounting for 34% of the identified proteins (Table 3 and Figure 2). Carboxylesterase, ecdysone oxidase, and fructosidase were the next most abundant proteins.

*H. zea* feeding induces tomato defense
The previous study showed the *H. zea* fed on tobacco significantly reduce tobacco defense by decrease the nicotine synthesis (Musser et al. 2002). To know how *H. zea* feeding affect tomato defense, we caged the *H. zea* on tomato leaf and compare the tomato defense by gene expression assay. Proteinase inhibitors 2 (*PIN2*) was chosen as a defense marker gene because it has been well characterized as a defense in tomato (Ryan 1990). The results showed *H. zea* feeding significantly induced *PIN2* expression in tomato leaves (Figure 3); after 24 h feeding, the *PIN2* expression was significantly higher than untreated control plants (ANOVA, $F_{(24 \text{hour})}=17.46$, $P=0.03$), however, there was no significant difference in *PIN2* expression induced between damage caused by caterpillars whose spinneret was intact and individuals with ablated spinnerets, which renders them unable to release saliva (Musser et al. 2002). But after 48 h, feeding by intact caterpillars significantly induced greater *PIN2* expression than feeding by ablated caterpillars ($F_{(48 \text{hour})}=36.25$, $P<0.01$). *H. zea* fed significantly induced *PIN2* expression on tomato leaf (figure 3a.).

To determine if saliva is affecting the genes in signaling pathways in the tomato plant, we also check quantify the gene expression after 24 hours insect feeding. The results showed that leaves fed by *H. zea* had significantly higher expression of *PPO* compared to untreated controls, and also the intact fed significantly induced *PPO* expression than the ablated fed leaves (figure 3b). While the “early responding” signaling genes *AOS*, *LOX* and *ARG* associated with the octadecanoid pathway after 24hours insect fed were not significantly induced by insect feeding, since these gene are JA biosynthesis gene and responding at every early stages (figure 3b).

**Caterpillar secretions affect tomato induced defense**

These data showed that *H. zea* saliva play an important role in induction of *PIN2*. To further confirm the role of saliva in inducing defense gene expression, we wounded Better Boy
and MicroTom leaves and applied saliva on the wounded site. The amount of salivary protein (~0.5 µg) used in these experiments is a very conservative estimate of how much is secreted during feeding (Peiffer and Felton 2005). The application of *H. zeae* saliva to wounded leaves significantly induced the expression of *PIN2* after 48 h in both Better Boy and MicroTom cultivars compared to the wounded control (ANOVA, *F*<sub>BB</sub> = 8.5, *p*=0.007; *F*<sub>MT</sub> = 70.09, *p*<0.001 Figure 4a). In contrast, we found that regurgitant did not significantly affect *PIN2* expression in micro-tom tomato leaves with 20µl regurgitant (ANOVA, *F*=2.71 *p*=0.115) compared to the wounded control (Figure 4b).

**Saliva and GOX affect tomato defense on different tissue**

Because GOX was the most abundant salivary protein and earlier reports showed that infiltration of tomato petioles with a H<sub>2</sub>O<sub>2</sub> generating system consisting of glucose and fungal glucose oxidase triggered *PIN2* expression (Orozco-Cardenas et al. 2001). To test how saliva and GOX affect tomato defense we tested the effect of GOX and saliva on *PIN2* expression in tomato leaves, green and red fruit, and flower tissues. We applied fungal GOX (0.4µg) at levels consistent with the amount secreted by caterpillars (0.5µg) (Peiffer and Felton 2005). The fungal GOX has very similar substrate specificity as the GOX from *H. zeae* salivary glands (Eichenseer et al. 1999) and thus serves as appropriate model for the insect enzyme.

Relative expression of the late responding gene *PIN2* was examined after 24 and 48 hours wounding and treatment. *H. zeae* saliva or fungal GOX had different effect on the gene expression on different tissue (Figure 5). Wounding with PBS, saliva or GOX both induce *PIN2* induction on the leaf at 24 and 48 hours. However the *H. zeae* saliva and GOX treatment significantly induced more *PIN2* expression than PBS treatment at both time points. At 48 hour, the *PIN2* expression in leaf treatment with saliva and GOX is significantly higher than at
24 hours time points, while the PBS treatments at the different times were not significantly different. The effect of induction of PIN2 expression by GOX and saliva was not significantly different at the two time points (ANOVA, F=20.70, p<0.001, Figure 5a). Treatments of green fruit with wounding and PBS, saliva or GOX showed PIN2 induction relative to the unwounded control. Compared to PBS, the saliva and fungal GOX treatments induced PIN2 expression in green fruit after wounding. At 24 hour, the saliva treatment induced higher PIN2 than the GOX treatment, while at 48 hour, the two treatments showed no significant differences between saliva and GOX (ANOVA, F=14.39, p<0.001, Figure 5b). But with red mature fruit, wounding with PBS, saliva or GOX treatment caused no significant PIN2 induction compared with non-treated fruits (ANOVA, F=4.43, p=0.058 Figure. 5c) Floral tissues had higher constitutive PIN2 expression level, but again there was no significant response to wounding or saliva in the flower tissues (Figure. 5d) (ANOVA, F=0.28, p=0.761).

**Trichome Induction by H. zea feeding or Saliva treatment**

Two weeks after H. zea fed, we counted the glandular trichome number on the newly growth expanded leaves. The results showed inset fed significantly induce the glandular trichome number on the newly growth leaves compared to unwounded leaves (425.6/ cm², SE=37.3). Compared to mechanical ablated insect fed, the glandular trichome number on the intact caterpillar fed were significantly induced (ANOVA, F=11.84, p<0.05) (Figure 6a). This result indicates that insect fed with secretion of saliva induce the glandular trichome number on the newly growth leaves.

To further examine the effect of caterpillar saliva on trichome induction, plants were wounded and saliva or GOX (20ng/µl) immediately applied to the wound site. After two weeks treatment, plants treated with saliva increase77.8% trichomes on the new growth leaf compared
to unwounded plants. GOX treatment also significantly induced trichome number on the new growth leaf (Figure 6b) (ANOVA, F=7.42, p<0.05).

Previous studies have shown glandular trichomes of *Lycopersicon* spp. is responsible for resistance to various insects (Gentile & Stoner 1968; Gentile *et al.* 1968; Dimock & Kennedy 1983; Kennedy & Sorenson 1985). Our Sigma Scan analysis showed after mechanical remove trichome, the leaf got more damage by *H. zea* 3rd larvae in 24 hours feeding compared to the control leaf (Figure 7a). The bioassay showed remove leaf trichome could significantly increase *H. zea* growth in 5 days feeding assay (Figure 7b). These results consistent with the previous study of glandular trichome play important role in tomato defense. The trichome hairs on leaf surface contain chemical that affect insect growth. It may also physically prohibit insect feeding.

**DISCUSSION**

Noctuid herbivores produce multiple secretions during feeding that have potential to mediate the induction of direct and indirect plant defenses (Felton 2008b). The typical mount of saliva released from a single larva is very tiny (~ 0.5nl) and regurgitant is about 5µl from each caterpillar. The regurgitant contain high quantity protein around 0.60µg in one caterpillar collections, there is no GOX activity was detected in the collection. The saliva contains 0.056 µg total protein in one caterpillar collections, but showed high GOX activity. The immunodetection confirmed when insect feed on leaf they release saliva which main composition is GOX.

One previous study showed highly polyphagous species possess relative high level of GOX compared to species with limited host range by examining the labial gland GOX activities in 23 families of Lepidoptera (85 species) (Eichenseer *et al.* 2010). Our results from shotgun proteomic analysis based on protein abundance showed that among total 33 proteins identified
(Table S1), glucose oxidase (GOX) was by far the most abundant protein in *H. zea* saliva, which accounting for 34% of the identified proteins. Since *H. zea* has wide host range from vegetable such as asparagus and watermelon to crops such as cotton and corn. This result confirmed the GOX is the main protein in *H. zea* saliva. And also carboxylesterase, ecdysone oxidase, and fructosidase were the next most abundant proteins in saliva. These proteins also need to be studied to know more functions in plant defense.

Salivary GOX has been reported in a few insect species which function as an effector in regulating plant defense in multiple plant species including *Nicotiana tabacum* (Musser et al. 2002), *Nicotiana attenuate* (Diezel et al. 2009), *Medicago truncatula* (Bede et al. 2006), and *Arabidopsis thaliana* (Weech et al. 2008). In one example with the tobacco budworm *Heliothis virescens* showed that the effect of saliva had an inhibitory effect on volatile induction and that both saliva and regurgitant were necessary to elicit the “volatile signature” of *H. virescens* feeding. Since proteinase inhibitor (*PIN2*) is the marker gene in JA pathway, in our current study we quantify relative *PIN2* gene expression to compare the different response of insect feeding or saliva treatment. The results showed that insect fed induce *PIN2* gene expression. The intact *H. zea* fed significantly induce *PIN2* gene expression compared with the spinneret ablated *H. zea* fed, which stop saliva secretions on leaf. This is different from the previous report of caterpillar saliva interferes with induce *Arabidopsis* defense (Weech et al. 2008). These data are particularly interesting because in tobacco, glucose oxidase acts an effector by suppressing the induction of nicotine in tobacco (Musser et al. 2005; Musser et al. 2002). When larvae feed on tomato they synthesize and secrete less glucose oxidase when feeding on tobacco (Peiffer and Felton 2005). This suggests that larvae may adjust their saliva or salivation to minimize induction and/or maximize suppression of induced defenses based on their host plant.
Since the main component of *H. zea* saliva is GOX, the results showed that wounding treatment with saliva or fungal GOX both induce *PIN2* expression compared control treatment. This is different from one previous study; the salivary gland homogenates had a small inhibitory effect on the induction of trypsin inhibitor activity in tomato (Musser et al. 2005). There were two differences in the methods employed in these studies. In the current study we used saliva directly collected from the spinneret, whereas in the previous paper salivary gland homogenates were used. The homogenates may contain materials that are not secreted during feeding. However the amounts of saliva that we can collect from the spinneret are small and less than the caterpillars normally would secrete during feeding (Peiffer and Felton 2005). Second, in the current study we tested the effects on induction of *PIN2*, whereas in the first paper total trypsin inhibitory activity was measured. While the regurgitant from *H. zea*, at the amounts tested, did not affect the expression of the defense gene *PIN2* compared to control. This is different from one previous study that oral secretion from the tobacco hornworm *Manduca sexta* elicits *PIN2* expression in the related species *Solanum tuberosum* (Korth and Dixon 1997). Although the exact amount of saliva that are secreted by larvae are not known, we used amounts that are consistent with what has been published in the literature for many lepidopteran species (Alborn et al. 1997; Qu et al. 2004; Rose and Tumlinson 2005).

Induced plant defense have mainly been studied in leaves (Karban et al. 2000) and only very few research have investigated induced plant defense on reproduction tissue. This disparity may be due to the allocation of resources to defense is determined by the cost of production. In this study we compared tomato leaves, flower, green fruit and red fruit response to wounding treatment, the results showed saliva and GOX treatment induce *PIN2* expression in leaves and green fruits. While the red fruits didn’t show any *PIN2* induction compared to unwounded fruits.
The expression of PIN2 in flowers showed significantly higher than in leaves and green fruits, but no induction by wounding and saliva or GOX treatment compared to unwounded flowers. This is consistent with the optimal defense theory (ODT) which explained the patterns of plant chemical defense and predict that induced defense should be rare in reproductive tissues (McCall and Karban 2006).

Trichome production is an important component of resistance against herbivorous insects (Antonious and Synder 1993; Levin 1973). While most plants produce trichomes constitutively, some plant species respond to damage by producing new leaves with an increased density of trichomes. Both artificial wounding and damage by herbivores can induce trichome production. The induction of trichome has been observed in both annual and perennial plants (Abdala-Roberts and Parra-Tabla 2005; Agrawal 1999). This study found insect feeding or wounding with saliva/GOX treatment could induce the newly growth leaf trichome density. This confirmed the saliva plays important role in inducing glandular trichome on the newly growth. The magnitude of the increase in trichome density is between 37%-139% in this study. Experimentally remove glandular trichome could increase H. zea consume more leaf foliage and significantly increase insect growth. This supports that trichome density negatively affects insect growth (Handley et al. 2005, Gentile et al. 1969, Dimock et al. 1982).

In summary, this study confirmed H. zea release saliva when they are feeding on tomato plants which play important role in regulating tomato and H. zea interactions. The saliva could induce tomato defense in leaf and green fruit, while flower and red fruit had no defense in response to the treatment. PIN2 as JA pathway maker gene was induced by insect feeding and wounding treatment. Trichomes function as defense insect feeding were highly induced by insect feeding or saliva and GOX treatment.
Table 1 Caterpillar oral secretion collection protein and GOX assay

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<tr>
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<th>Regurgitant</th>
<th>Saliva</th>
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<tr>
<td>Total Protein/Caterpillar</td>
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<td>0.56µg</td>
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<tr>
<td>GOX activity</td>
<td>0</td>
<td>1.785714 mol/min/mg</td>
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Table 2 Primers used for real-time PCR assays of relative expression

<table>
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<tr>
<th>Primer name</th>
<th>Gene Name/ Accession number</th>
<th>Primer sequence (5'-3&quot;, forward/reverse)</th>
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<tbody>
<tr>
<td>LOX</td>
<td>Lipoxigenase D/u37840</td>
<td>F: GTT CAT GGC CGT GGT TGA CAC ATT / R : TGG TAA TAC ACC AGC ACC ACA CCT</td>
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<tr>
<td>AOS</td>
<td>Allene Oxide Synthase/af230371</td>
<td>F: ACC CGT TTA GCA AAC GAG ATC CGA / R: CGT TGC AAA TGG TTG GTA CCC GAA</td>
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<tr>
<td>ARG</td>
<td>Arginase/ AY656838</td>
<td>F: TGG TGA AGG TGT AAA GGG CGT GTA / R: TTA CCA GCT TCG CAG CAA CCA TTG</td>
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<tr>
<td>PPO</td>
<td>Polyphenol oxidase F/ Z12838</td>
<td>F: ATG TGG ACA GGA TGT GGA ACG AGT R: ACT TTC ACG CGG TAA GGG TTA CGA</td>
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<td>PIN2</td>
<td>Wound inducible proteinase inhibitor 2/ K03291</td>
<td>F: GGA TTT AGC GGA CCT CCT TCT G / R: ATG CCA AGG CTT GTA CTA GAG AAT G</td>
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<tr>
<td>Ubi</td>
<td>Ubiquitin/ X58253</td>
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Table 3. Proteomic Identification of Salivary Proteins in *H. zea*

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<th>Organism</th>
<th>NCBI Accession</th>
<th># of Peptides</th>
<th>Total Ion Score</th>
<th>MW</th>
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<td>215982092</td>
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<td>2 glucose oxidase-like enzyme</td>
<td><em>Helicoverpa armigera</em></td>
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<td>186909546</td>
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<td>3 carboxyl/choline esterase CCE016d</td>
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<td>4 putative ec dysone oxidase</td>
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<td>219815604</td>
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<td>5 Fructosidase</td>
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<td>156968287</td>
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<td>6 epoxide hydrolase</td>
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<td>8 aryl-alcohol oxidase precursor, putative</td>
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FIGURE LEGENDS

**Fig. 1. Saliva GOX detection on MicroTom-leaves**  The Micro-tom tomato leaf were fed by ablated and intact *H. zea* overnight, Then after transfer to nitrocellulose and superblock, and incubated with anti-GOX, detected the GOX spread on leaf with vector ABC Elite and Vector a). Ablated *H. zea* fed leaf b). Intact *H. zea* fed leaf

**Fig. 2. Relative peptide abundance of *H. zea* salivary proteins**  Shotgun proteomic analysis of secreted salivary proteins showed most abundant protein accounting for the total identified proteins. Glucose oxidase (GOX) was by far the most abundant protein accounting for 34% of the identified proteins

**Fig. 3. PIN2 relative expression of PIN2 in tomato leaves 24 and 48 hours after feeding, real-time PCR compare PIN2 gene expression.**  Data shows mean relative expression ±SE. Letters represent significant differences between PBS and saliva treated samples (P<0.05) by Fisher’s comparison. (ANOVA, F(24hour)=17.46, P=0.03; F(48hour)=7.51, P=0.023).

**Fig. 4. Compare relative expression of defense genes 24 hours after wounding and application of *H. zea* saliva from 10 larvae on tomato leaf.** a) Treatment on different varieties Better boy and Micro-tom tomato leaf both induce PIN2 induction compared to PBS treatment (ANOVA, F_{Better boy} = 8.5, p=0.007; F_{MicroTom} = 70.09, p<0.001). b) Regurgitant treatment on the Micro-Tom leaf didn’t significantly induce PIN2 relative expression compared to PBS treatment (ANOVA, F=2.71, p=0.115). Data shows mean relative expression ±SE. Asterisk represent significant differences between PBS and saliva treated samples (P<0.05) by Fisher’s –LSD comparison
Fig. 5. Relative expression of PIN2 on different MicroTom tissues 24 and 48 hrs after wounding and treatment with PBS buffer, saliva or fungal GOX a) PIN2 significantly induced by saliva and GOX treatment compared to PBS buffer on leaf; b) PIN2 significantly induced by saliva and GOX treatment compared to PBS buffer on Green fruit; c) No PIN2 induction on red fruit, d) Flower Receptacle wounding with treatment didn’t show PIN2 induction. Error bars represent mean ± SE.

Fig. 6. Trichome induction by wounding treatment or insect feeding  Average number of trichomes on new growth leaf after wounding or insect feeding a) Ablated and Non-ablated insect feeding increase the new growth leaf trichome number b) Wounding and treatment with H. zea saliva or fungal glucose oxidase (20ng/µl), non-wounding and wounding with PBS buffer treatment as control. Data shown are error bars represent mean ± SE.

Fig. 7. Trichome significantly affect H. zea feeding and growth a) After removing the leaf surface trichomes, the leaf damage significantly increased. b) The leaf with trichome removed significantly increased H. zea growth.
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Fig. 3. Relative gene expression in tomato leaves after *H. zea* feeding. Cage *H. zea* on the 4th leaf for 6 hours feeding, after 24 and 48 hours, real-time PCR compare the gene expression. Ablated fed means the 5th instar larvae spinneret was ablated, intact fed means regular insect feeding, control means empty cage on the leaf. a) *PIN2* relative expression by ablated and intact fed with 24h and 48h. b) LOX, AOS, ARG and PPOF relative expression after 24 hour *H. zea* fed. Data shows mean relative expression ±SE.
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Reference


Agrawal AA (1999) Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80: 1713-1723


Chippendale G (1970) Development of artificial diets for rearing the *Angoumois* grain moth. J.Economic Entomology 63: 844-848


Spiteller D, Dettner K, Boland W (2000) Gut bacteria may be involved in interactions between plants, herbivores and their predators: Microbial biosynthesis of N-acylglutamine surfactants as elicitors of plant volatiles. Biological Chemistry 381: 755-762


Chapter III
Role of Trichomes in Defense against Herbivores:

Comparison of Herbivore Response to Woolly and Hairless Trichome Mutants in Tomato
(Solanum lycopersicum)

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Abstract

Trichomes contribute to plant resistance against herbivory by physical and chemical deterrents. To better understand their role in plant defense, we systemically studied trichome morphology, chemical composition and the response of insect herbivores *Helicoverpa zea* and *Leptinotarsa decemlineata* (Colorado potato beetle) on the tomato *hairless* (*hl*) and hairy (*woolly*) mutants. *Hairless* mutants showed reduced number of twisted glandular trichomes (type I, IV, VI and VII) on leaf and stem, while *woolly* mutants showed high density of non-glandular trichomes (type II, III and V) but only on the leaf. In both mutants, trichome densities were induced by methyl jasmonate (MeJA), but the types of trichomes present were not affected by MeJA treatment. Glandular trichomes contained high levels of monoterpenes and sesquiterpenes. A similar pattern of transcript accumulation was observed for monoterpene *MTS1* (=TPS5) and sesquiterpene synthase *SST1* (=TPS9) genes in trichomes. While high density of non-glandular trichome on leaves negatively influenced CPB feeding behavior and growth, it stimulated *H. zea* growth. High glandular trichome density impaired *H. zea* growth, but had no effect on CPB. Quantitative real-time polymerase chain reaction (qRT-PCR) showed that glandular trichomes highly express protein inhibitors (*PIN2*), polyphenol oxidase (*PPOF*) and hydroperoxide lyase (*HPL*) when compared to non-glandular trichomes. The SlCycB2 gene, which participates in woolly trichome formation, was highly expressed in the *woolly* mutant trichomes. *PIN2* in trichomes was highly induced by insect feeding in both mutant and wild type plants. Thus both the densities of trichomes and the chemical defenses residing in the trichomes are inducible.

Key words: jasmonate, plant defenses, induced defenses, herbivores, *Leptinotarsa decemlineata, Helicoverpa zea*
Abbreviations:

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Introduction

Trichomes are hair-like protuberances that develop from the aerial epidermis on leaves, stems and other organs on many plant species (Creelman and Mullet 1995; Duffey 1986; Kang et al. 2010a; Kang et al. 2010b; Reeves 1977; Steffens and Walters 1991; Wagner 1991). Trichomes are normally classified as either glandular or non-glandular and may vary in size, shape, number of cells, morphology, and chemical composition (Goffreda et al. 1989; Kang et al. 2010a; Kang et al. 2010b; Schilmiller et al. 2010; Stout et al. 2002). Numerous studies have shown that trichomes serve multiple functions including defense against herbivores by interfering with their movement and/or by direct toxicity through chemicals they produce and/or release (Arimura et al. 2005; Dimock et al. 1982; Kang et al. 2010a; Kang et al. 2010b; Kennedy 2003; Peiffer et al. 2009; Wagner 1991). Trichome-based plant resistance holds potential to improve sustainability of insect pest management by reducing pesticide use and decreasing the likelihood that pesticide resistance will develop (Dalin and Björkman 2003; Radhika et al. 2010; Simmons and Gurr 2004).

High densities of foliar trichomes may prevent feeding damage by herbivores (Handley et al. 2005; Horgan et al. 2009; Kessler et al. 2011). Several studies have shown that herbivore feeding or mechanical damage to leaves leads to newly formed leaves with higher densities of trichomes (Agrawal et al. 2002; Agren and Schemske 1994; Andrade et al. 2005; Stratmann and Ryan 1997; Traw and Dawson 2002a). In some instances, jasmonic acid regulates trichome production and plant defense (Halitschke et al. 2008; Li et al. 2004; Traw and Bergelson 2003). In tomato, application of methyl jasmonate (MeJA) increases densities of glandular trichomes on new leaves (Boughton et al. 2005). The inducibility of trichome density is ecologically significant because trichome density can negatively influence herbivore populations (Agrawal
While herbivory or wounding is known to affect the quantitative variation in trichome density, it is unknown if herbivory may also influence qualitative variation by altering the expression of defensive chemistry within glandular trichomes.

Because tomato (*Solanum lycopersicum*) is an economically important crop, it serves as a model for studying plant defenses against herbivores and diseases (Green and Ryan 1972; Howe and Jander 2008; Li et al. 1999; Mirnezhad et al. 2010; O'Donnell et al. 1996). Decades of research on tomato has shown that jasmonate signaling (JA) plays an important role in plant defense signaling against insect herbivory (Bostock 2005; Felton et al. 1994; Howe and Jander 2008; Thaler et al. 2001). Trichomes in cultivated tomato and related wild species have been subjects of intense study for many decades (Kennedy 2003; Peiffer et al. 2009; Steffens and Walters 1991). Unlike Arabidopsis, which only has non-glandular trichomes (Seo et al. 1999), trichomes in *Solanum* are highly diverse in morphology and chemistry. Tomato foliar trichomes are categorized as types I-VII, with types I, IV, VI and VII being glandular and types II, III and V being non-glandular (Kang et al. 2010b; Luckwill 1943). The major distinction between non-glandular and glandular is that glandular trichomes have heads containing various sticky and/or toxic chemicals that may poison or repel herbivores (Schilmiller et al. 2010). Non-glandular trichomes do not have heads and are thought to mainly function in defense by physically hindering insect feeding behavior and movements (Higgins et al. 2007).

Insect behavior can be dramatically influenced when trichomes obstructs movement across the plant surface (Radhika et al. 2010; Simmons and Gurr 2005). Moreover, glandular trichomes may have profound effects on herbivore performance (i.e., growth, survival and fecundity) and host-plant-selection behavior (Duffey 1986; Higgins et al. 2007; Kennedy 2003; Neill et al. 2002; Pelletier and Dutheil 2006). The most remarkable feature of tomato glandular
trichomes is their capacity to produce and secrete a wide variety of plant secondary compounds including terpenoids (Hogenhout and Bos 2011; Kang et al. 2010b; Karban and Baldwin 1997; Ma et al. 2011; Panizzi 1997; Schilmiller et al. 2010), phenolics (Gang et al. 2001), sucrose esters, methyl ketones (Fridman et al. 2005) and organic acids (Toth et al. 2005). By studying isolated glands, many of genes involved in the biosynthesis of the chemicals have been characterized, including the roles of monoterpene \textit{MTS1(=TPS5)} and sesquiterpene \textit{SST1(=TPS9)} synthesis genes in regulating terpene biosynthesis (Besser et al. 2009).

Several methods have been used to study trichome functions in defense including manipulative and genetic methods. For instance, the experimental removal of tomato glandular trichome heads and exudates significantly decreased the mortality of aphids, the tomato fruitworm \textit{Helicoverpa zea}, and the Colorado potato beetle \textit{Leptinotarsa decemlineata} (Dimock et al. 1982; Kennedy 2003; Simmons and Gurr 2004). However, such mechanical treatments of the leaf are likely to induce JA-regulated defenses (Peiffer et al. 2009) and can confound interpretation of results by altering the leaf surface and chemistry in ways unrelated to natural variation in trichome density. The use of mutants is a more rigorous and less ambiguous experimental approach for deciphering the roles of different trichome types in resistance against herbivores (Kang et al. 2010a; Kang et al. 2010b).

In tomato, the effect of trichome-based resistance on insect herbivores has mostly focused on glandular trichomes and there has been considerably less study on the role of non-glandular trichomes against herbivores (Kang et al. 2010a; Kang et al. 2010b). Because most cultivars possess both glandular and non-glandular trichome, it is difficult to separate trichome function(s) in these commercial varieties. In this current study, we use two mutants with differing trichome types to examine the role of glandular and non-glandular trichomes in
resistance to the solanaceous specialist *L. decemlineata* and the generalist *H. zea*. In addition to characterizing the morphologies and densities of the trichomes in these mutants, we also report their terpene composition. We hypothesize that both glandular and non-glandular trichome phenotypes will provide different contributions to defense against the herbivorous insect species.

**Materials and Methods**

**Plants and Insects**

Tomato (*S. lycopersicum*) hairless mutant (*hl*, LA3556) and wild type Alisa Craig (AC, accession number LA2838), hairy mutant (*woolly*, LA0258) and wild type Rutgers (RU, accession number LA1090) were used in all experiments. Seeds were originally obtained from Tomato Genetics Resource Center (University of California, Davis, CA, USA). Seedlings were grown as described previously (Peiffer and Felton 2005) in Metromix 400 potting mix (Griffin Greenhouse & Nursery Supplies Tewksbury, MA) in greenhouse at Penn State University, University Park, PA, USA. The greenhouse was maintained on a 16-h photoperiod.

Tomato fruitworm (*Helicoverpa zea*) and Colorado potato beetle (=CPB) (*Leptinotarsa decemlineata*) were reared in the Entomology Department, Penn State University (University Park PA, USA). *Helicoverpa zea* eggs were purchased from BioServ (Frenchtown, NJ) and reared on a wheat germ and casein-based artificial diet (Bansal et al. 2011) with ingredients from Bioserv. Colorado potato beetle was collected from tomato plants in Centre County PA and reared on tomato plants as described previously (Chung and Felton 2011).

**Morphology and density of different type trichome on mutant**

At four-leaf stage, ten plants of each cultivar were sampled to compare trichome morphology and density of the trichome on leaf. Two leaf discs from the youngest fully
expanded leaf were cut from each side of the mid-vein. Trichome numbers were counted under a light microscope on each leaf disc. The density of trichomes was calculated as the number per disc/cm².

Scanning electron microscopy (SEM) was performed to compare the morphology of trichomes on different mutants and followed the protocol described in Kang et al (Kang et al. 2010b). Briefly, tissues were fixed overnight in a solution of 2.5% paraformaldehyde, 2.5% glutaraldehyde buffered with 0.1M sodium cacodylate, pH 7.0. Samples were dehydrated in a graduated ethanol series, critical point dried, then mounted, and sputter coated. Samples were then examined with a 20 kV accelerating voltage with a JEOL JSM5400 microscope (Tokyo, Japan).

**Trichome purification and Real-time PCR**

Trichomes were purified following well-established methods with slight modifications (Peiffer et al. 2009). To isolate trichomes, leaflets/stems were removed, placed in a 50ml conical tube containing 1g glass beads (diameter, 4mm) (Kimble chase, Vineland, NJ, USA) and liquid N₂, and the tube was shaken vigorously to shear trichomes off the leaf/stem. We then poured the slurry through a 1mm strainer for non-glandular trichomes and rinsed with liquid N₂ while glandular trichomes need go through 100µm strainer again and rinsed with liquid N₂. The purified trichome preparation was used for chemical analyses and quantification of trichome specific gene expression.

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to compare gene expression. One hundred mg of purified trichomes were homogenized in liquid nitrogen and total RNA was purified with an RNeasy Plus Mini-kit which can remove genomic DNA (Qiagen, Valencia, CA, USA). A High Capacity cDNA Reverse Transcription kit (Applied Biosystems,
Foster City, CA,) was used for cDNA synthesis. All qRT-PCR reactions used Power SYBR
Green PCR Master Mix and ran on 7500 Fast Real-Time PCR system (Applied Biosystems)
following standard protocols (10min at 95°C, with 40 cycles of 15s at 95°C and 60s at 60°C).
Relative quantifications were calculated with wild type RU leaf RNA as the reference group. The
ubiquitin gene was used as the housekeeping gene to normalize $2^{-\Delta\Delta C(T)}$ (Rotenberg et al. 2006).
To validate this analysis method, primer efficiency was analyzed by comparing the normalized
$C(T)$ values of five serial dilutions of cDNA. Melting curve analysis was conducted to confirm
the specificity of the primers. Three replicates for each varieties. The relative gene expression
value was analyzed by one way ANOVA followed by Fisher-LSD to do the multi-comparisons.
The primers used are shown in Table 1.

**Terpene Analyses**

Because terpenes have been reported to be important phytochemical components of
tomato trichome-based resistance (Kang et al. 2010a; Kang et al. 2010b; Wagner 1991), we
explored terpene content using two methods. In the first method, we dissolved 100 mg of
purified leaf or stem trichomes directly in 1ml methyl tertiary-butyl ether (MTBE) buffer. For
the second method, we dipped leaves into MTBE to remove surface terpenes. After weighing
two leaves sampled from youngest fully expanded, the leaves were incubated at room
temperature in 1.0 ml of (MTBE) for three minutes with gentle shaking. For both assays, we
collected three replicate samples per treatment.

For all trichome extractions, we added to each sample 200 ng $n$-octane and 400 ng nonyl
acetate as external standards. Trichome extracts were injected in 1 µl aliquots into an Agilent
model 7890 gas chromatograph (GC) fitted with a flame ionization detector, using a splitless

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injector held at 250ºC. The column (HP-5; 30 m long × 320 µm interior diameter × 0.25 µm film thickness; J&W Scientific, Folsom, CA) was maintained at 35º C for 1 min sec, then ramped at 5º C per min to 250º C. Quantifications of compounds were made relative to the nonyl acetate standard using Chem Station software (Agilent Technologies, Wilmington, DE). Identifications of trichome components were made with GC-MS in electron ionization mode comparing retention times and spectra with that of commercial standards.

**Trichome induction by MeJA and Bioassay**

To compare different trichome responses to MeJA treatment, we treated plants with 3.3 mM MeJA (Sigma, St. Louis, MO, USA) in 0.8% ethanol at 4th leaf stage. Two weeks after the spray treatment, we counted trichomes on the youngest fully expanded leaf as previously described. From the same cohort of plants, the two youngest leaves were collected for bioassays with *H. zea* and CPB. Thirty insects per treatment were used for the bioassay.

Bioassays using the mutants were conducted to compare how different types of trichomes affect insect growth. Detached leaves were used for the bioassay. Newly hatched *H. zea* larvae were reared on artificial diet for two days before transfer to tomato leaf for bioassay. Newly hatched CPB larvae were put on tomato leaf directly in a standard 1 oz. cup (BioServ, Frenchtown, NJ) with 2% agar on the bottom to maintain leaf moisture. Insects were kept at 27º C, with a 16:8 L:D photoperiod in an incubator. Thirty insects were used for each bioassay. After 5 days, larval weights were recorded using a precision balance and were statistically analyzed by one-way ANOVA followed by Fisher-LSD for multiple comparisons.

To compare how different trichome type affects insect feeding behavior, we used 3rd instar *H. zea* or 2nd instar CPB larvae on a detached tomato leaf. We recorded in a ten minute
interval the time spent feeding during our observation under microscope. Fifteen larvae were observed for each species and genotype.

We also tested whether the mutants would impact the oviposition of CPB or *H. zea* moths. Ten mated female *H. zea* moths or ten randomly selected CPB adults were released in cages containing one plant from each of the four genotypes. After 24 h, we examined the plants thoroughly and counted the number of eggs on each plant. The experiment was replicated in eight cages. The average number of eggs on plants was compared on different mutant and wild type plants through one way ANOVA.

**Plant response to insect feeding**

At the four-leaf stage, 5th instar *H. zea* were individually caged on the fourth leaf for 6 h, control plants had empty cages. Experiment was replicated nine times. Larvae were removed and after 24 h, the caged leaf was sampled for RNA extraction and qRT-PCR as previous described to compare *PIN2* relative expression with control leaf. Ubiquitin gene was used as internal control to normalize data.

**Statistical analyses**

Data was analyzed using general linear models for analysis of variance (ANOVA) and where appropriate with post-hoc comparison of means using the Fisher-LSD means separation test using Minitab (University Park, PA).

**Results**

**Morphology and density of trichome on mutants and induction by MeJA**

As observed with light microscopy and SEM, the morphology and trichome densities of the mutants and their respective wild types were highly variable (Fig. 1, 2). The *woolly* mutant
had abundant trichomes on leaves (Fig. 1 a), but primarily non-glandular types II, III and V. Trichome types II and III were similar in length, but differed in their base, which was multicellular under type II trichomes, but unicellular base under type III (Fig. 2 a). Type V trichomes were shorter and had unicellular bases (Fig. 2 a). While trichomes on the woolly mutant stem possessed both glandular and non-glandular trichomes, but were dominated by non-glandular trichomes on leaf (Fig. 1 a, c)

The hl mutant, which has been described in Kang et al (2010a), had fewer foliar trichomes than wild-type leaves. These mutant leaves had normal type I trichomes but most others were highly twisted and swollen (Fig. 1 g). Type VI glandular trichomes have short neck cells that connect to the four-celled gland, and they showed irregular patterns and low density on the leaf surface (Fig.1 g and Fig. 2 b). The trichomes on stems of the hl mutant showed similar twisted, swollen morphology and lower density (Fig.1 g, i). Both wild types AC and RU leaves and stems possessed all types of trichomes including glandular and non-glandular, but type VI glandular trichomes were the most abundant on leaves and stems (Fig.1 d, f, j, i & Fig. 2 c, d).

Densities of trichomes on the mutants were significantly different than their respective wild type genotypes. The mutant hl displayed twisted and low density of glandular trichomes on leaf surface; the glandular trichome density was reduced by 57% compared to wild AC plants and no non-glandular trichome on the leaf surface. The density of the twisted glandular trichomes on hl was induced by MeJA treatment; twisted glandular trichomes on newly growth leaf were increased 85.8% after MeJA treatment. While the woolly mutant showed high density of non-glandular trichomes with very few glandular trichome on leaf surface compared to wild type RU plants (Fig. 3 a, b). The non-glandular trichomes on the woolly mutant also were induced by MeJA; non-glandular trichome numbers increased 37.8% following treatment. Also
the non-glandular trichome length was increased 71.4% by MeJA compared to untreated plants (Fig. 3 c). The glandular trichome densities on the RU and AC leaf surface were increased 48.1% and 64.8% by MeJA (Fig. 3 b). Both type trichome densities on the leaf are induced by MeJA treatment.

**Accumulation of terpenes in different trichomes**

Glandular trichomes function either as sites for synthesis and/or the site of release for various defensive compounds. Previous reports indicate that monoterpenes and sesquiterpenes are produced in leaf trichomes (Fridman et al. 2005). Because there is significant variability in the morphology and density of trichomes on the leaf and stem of mutants, we compared the terpenoid composition of trichomes in these mutant lines to their wild types. Trichomes produced a mixture of several monoterpenes with β-phellandrene, (Z)-3-hexenyl acetate and α/β-pinene being the main constituents. Monoterpenes in leaf trichomes were significantly more abundant than in stem trichomes (Fig. 5 a). In the *hl* mutant, all the monoterpenes in leaf trichomes were significantly higher than in wild type trichomes. Compared to the *hl* mutant and wild type, *woolly* mutants showed similar monoterpene profiles, with β-phellandrene and (Z)-3-hexenyl acetate as the main constituents, whereas β-pinene was not detected in the *woolly* mutant leaf trichomes (Table S1). The total monoterpenes in the *woolly* leaf mutant were significantly less than in the wild type (Fig. 5 a; Table S1). These results indicate that foliar trichome monoterpane levels were significantly higher than in stems, and also monoterpenes are mainly produced in glandular trichomes.

The profile of sesquiterpenes in trichomes showed the main components are ε-2-hexenylbutyrate, β-caryophyllene and z-jasmone (Table S2). Compared to monoterpenes, the quantity of sesquiterpenes was significantly less in the both leaf and stem trichomes (Fig. 5 a, b).
Most sesquiterpene levels in stem trichomes were less than foliar trichomes. Also the z-3-hexenyl butyrate, α-humulene and β-farnesene weren’t detected in both hl and woolly stem trichomes (Table S2). The hl glandular trichome sesquiterpene level showed higher quantity than wild type plants, while woolly mutant non-glandular trichome showed lower quantity than wild type plants.

Analysis of leaf dip extracts also showed that the hl and woolly mutants had less monoterpenes and sesquiterpenes than their respective wild types AC and RU. This is likely due to decreased trichome density on hl plants relative to wild types, and the lack of glandular trichomes on woolly mutants (Fig. 5 c). Results from both extraction methods indicate that glandular trichomes are the main source of monoterpenes and sesquiterpenes. Kang et al (2010) reports on the trichome terpenoids analysis showed less β-phellandrene and other sesquiterpenes in type VI trichomes. These results showed that compared to glandular trichomes, the non-glandular trichomes accumulate less of these chemicals in the trichomes. Although the trichomes on the hl mutant showed distorted morphology, it did not significantly affect the chemical composition in the leaf trichomes.

**Comparison of gene expression in different tomato trichomes**

Trichomes on many solanaceous species are known to express terpene synthases and produce a variety of terpenes (Seo et al. 1999). Previous study showed that trichome specific transcripts play a role in controlling terpenoid production (Besser et al. 2009). In this study, we examined the expression of two genes encoding terpenoid synthesis: $MTS1 (=TPS5)$ which encodes for monoterpane synthase and $SST1 (=TPS9)$ which encodes for sesquiterpene synthase. Transcript levels measured by qRT-PCR showed that $MTS1 (=TPS5)$ and $SST1 (=TPS9)$ are highly expressed in glandular trichomes (Fig. 6). The relative expression of $MTS1 (=TPS5)$ and
$SST1 (=TPS9)$ in the *woolly* mutant is 9.2 and 3.6-fold lower than the wild type, while in the *hl* mutant $MTS1 (=TPS5)$ and $SST1 (=TPS9)$ are more highly expressed than wild type (Fig. 6 a, b). These results are consistent with the monoterpane and sesquiterpene levels in the trichomes.

In addition to the defense provided by terpenes, Proteinase inhibitors are another class of defensive proteins in tomato and can accumulate in many plant tissues (Kang et al. 2010a; Kang et al. 2010b; Ryan 1990). Our study showed the relative expression of $PIN2$ is highly expressed in *hl* trichomes compared to the non-glandular *woolly* trichomes (Fig. 6 c.). Polyphenol oxidase ($PPO$) is another important defensive protein which is encoded by seven-member gene family and high expressed tomato trichomes (Chen et al. 2010; Thipyapong and Steffens 1997). Our results showed that $PPOF$ gene is highly expressed in glandular trichomes, but has very low expression in the *woolly* mutant, which lacks glandular trichomes. Hydroperoxide lyase ($HPL$) cleaves C18-lipid hydroperoxides to form a C6 aldehyde and a 12 carbon o xoacid. HPL activity has been found in a variety of plants and is associated with plant development and defense. In our experiments, compared with wild type leaf RNA, the $HPL$ was highly expressed in glandular trichomes, but less expressed in non-glandular trichomes (Fig. 6 e). Recent study showed that the B-type cyclin ($SlCycB2$) gene participates in trichome formation (Chen et al. 2008), and our study showed that $SlCycB2$ was highly expressed in *woolly* mutant trichome, but with lower expression in *hl* mutant (Fig 6 d). This result confirmed that $SlCycB2$ plays an important role in non-glandular trichome formation.

**Effects of mutants on insect growth and oviposition**

Because the morphology, density and chemical composition of trichomes are different between mutants and their wild types, these varieties may differ in their resistance to insect herbivores. Bioassays showed that *H. zea* larval grew significantly better on *hl* mutants than on
wild type AC plants indicating that glandular trichomes are an important source of resistance to this insect. Both *hl* mutant and wild type glandular trichomes are high induced by MeJA treatment and the larval growth on induced leaves was significantly reduced on both *hl* and the AC wild type plants (Fig. 7 a). While *woolly* mutant had high density of non-glandular trichomes, *H. zea* growth was significantly better on this mutant compared to wild type RU plants. Again, MeJA treatment enhanced resistance to *H. zea* for both woolly mutant and wild type plants (Fig. 7 a). This result confirmed glandular trichome density is an important factor in resistance to *H. zea*.

In contrast to *H. zea*, Colorado potato beetle (CPB) larvae showed no significant differences in larval growth on *hl* mutants compared to wild type (Fig. 7 b). These data indicate that the density of glandular trichomes found on the wild type is not an important mediator of larval growth in CPB. Additionally, MeJA treatment did not affect larval growth on newly formed leaves for the *hl* mutant or its AC wild type. Conversely, the *woolly* mutants significantly reduced CPB growth compared to wild type plants (Fig. 7 b), indicating that the very high densities of non-glandular trichomes on this mutant strongly influenced CPB growth.

To determine if the larval growth data relate to feeding behavior, we recorded the time larval *H. zea* and CPB spent feeding on mutant and wild-type plants. The duration of CPB feeding on the *woolly* mutant was significantly lower than the amount of time spent feeding on wild-type leaves indicating that growth reduction can be, in part, explained by a reduction in feeding due to the high density of non-glandular trichomes. The amount of time *H. zea* spent feeding was not influenced by non-glandular trichomes on *woolly* mutants, but fewer glandular trichomes on woolly leaves appeared to allow *H. zea* to feed longer than on wild type plants. During our observations of *H. zea* feeding, we found that larvae preferentially ingested non-
glandular trichomes prior to feeding on leaf mesophyll. There was no evidence that larvae later regurgitated the non-glandular trichomes. The CPB do not preferentially feed on the trichomes and it appears that the trichomes interfere with their access to the leaf (Fig. 7c).

Because trichomes have been reported to influence insect oviposition (Heinz and Zalom 1995; Horgan et al. 2009; Kessler et al. 2011), we conducted this experiment to determine if tomato trichomes influence ovipositional choice for both insect species. We found no observable differences in ovipositional choice for the different plant varieties (Fig. S1). *H. zea* moths laid eggs randomly on the adaxial leaf surface of all varieties and CPB laid eggs in clumps of 5-20 on the adaxial leaf surface equally across varieties (Fig. S2).

**PIN2 Induction by insect feeding**

Because trichome expression has been shown to be partly dependent upon a functional jasmonate signaling pathway (Peiffer et al. 2009), we tested if induction of the JA-regulated defense gene *PIN2* was influenced by *H. zea* feeding. In all genotypes, the expression of *PIN2* was significantly induced by feeding compared with their unwounded plants. However, no differences in levels of *PIN2* induction were detected among the different mutants and wild type plants (Fig. 8). These results indicate that JA signaling is activated by insect feeding damage in both mutants and wild type plants and suggest that differences in insect growth on the mutants were likely not due to significant impairments in jasmonate signaling.

**Discussion**

Trichome-based resistance in tomato plants offers a feasible approach to reduce pesticide applications (Simmons and Gurr 2004). Trichome morphology, density and chemical composition are important mechanisms of defense to prevent or decrease herbivore damage. The morphology and density of leaf trichomes vary considerably among plant species and may also
vary among populations and within individual plants (Dalin and Björkman 2003). However, in plants such as tomato which possess a variety of trichome types, these specialized cell types can be difficult to isolate separately to study the function of each type of trichome. Thus, the use of mutants provides a good tool to study trichome function. The hl mutant was described as hairless, and the phenotypic expression of this mutant at the microscopic level shows a characteristic twisting and shortening of trichomes (Kang et al. 2010b; Reeves 1977). The woolly mutant was described as hairy with a high density of type II and III non-glandular trichomes on leaf surface.

Numerous studies have shown that trichomes are capable of synthesizing and either storing or secreting large amounts of specialized metabolites that could influence their interaction with herbivores (Hogenhout and Bos 2011; Schilmiller et al. 2010). Chemical profiles of type VI trichome in hl mutant showed similar profiles of monoterpenes and sesquiterpenes with wild type plants, but with less β-phellandrene, β-caryophyllene and α-humulene (Kang et al. 2010b). In our experiment, we collected both types of trichome from the hl mutant, but the profiles of chemical composition were different from previous results. The β-phellandrene, β-caryophyllene and α-humulene levels were higher in hl trichomes than in the AC wild type trichomes. The leaf dip method showed the same pattern with previous results (Kang et al. 2010a; Kang et al. 2010b). These results indicated that although the trichomes were distorted in the hl mutant, the chemical accumulation in the twisted head cells was not affected. Not surprisingly the woolly mutant trichomes showed significantly low monoterpenes and sesquiterpenes confirming that that the glandular trichome is the main source of terpenes. Our results also indicated that the foliar trichomes had higher levels of monoterpenes and sesquiterpenes than the stem trichomes.
Our results indicate that both glandular and non-glandular trichomes function in insect defense. The non-choice bioassay showed the growth of *H. zea* larvae was substantially improved by feeding on the *hl* genotype compared to the wild type parent (cv. Alisa Craig). The bioassay results with the *hl* mutant are consistent with Kang (2010a) who observed similar effects with another caterpillar species, *Manduca sexta*. These results show that caterpillar growth is compromised by the presence of glandular trichomes which contain an arsenal of chemical defenses (e.g., terpenes, polyphenol oxidase, etc.) that may entrap small larvae as they attempt to move on the leaf surface (Simmons and Gurr 2004). Moreover, the feeding time of *H. zea* was significantly longer on the *hl* genotype compared to the parent wild type (cv. Alisa Craig). Larval *H. zea* spent more time feeding and had enhanced growth on the *woolly* genotype compared to the parent wild type (cv. Rutgers). These results indicate that the absence of glandular trichomes combined with a high density of non-glandular trichomes greatly enhances *H. zea* larval growth. We observed that larvae preferentially ingested the non-glandular trichomes prior to feeding on the remaining leaf tissue. By comparison, when *H. zea* feed on leaves with high densities of glandular trichomes, they frequently are observed to remove the trichomes and egest them (personal observations). Our results are quite different from the recent finding in *Nicotiana attenuata* where neonate *M. sexta* preferentially consume tobacco glandular trichomes as their first meal (Celorio-Mancera et al. 2011).

The results with the specialist beetle *L. decemlineata* were markedly different from what we observed with *H. zea*. The *L. decemlineata* growth was not improved when larvae fed on the *hl* genotype compared to the wild type parent. The *hl* mutant possesses a low density of glandular trichomes and reduced total terpenes in the leaves compared to the wild type plants. Although beetle larvae were observed to feed for longer bouts on the *hl* mutant leaf compared to
wild plants, this did not result in improved growth during the time course of our bioassay. Our bioassay results were different from Kang et al (2010b). They investigated resistance of the odorless-2 (od-2) mutant and found that larval growth of *L. decemlineata* was significantly increased on od-2 mutant compared to wild type plants. Although od-2 possesses a normal glandular trichome phenotype, the levels of terpenes and flavonoids in the trichomes are compromised. In our study we used *L. decemlineata* which originated from tomato fields and has been reared solely on tomato. Perhaps our colony is better adapted to tomato and is not as sensitive to the level of terpenes and flavonoids in the glandular trichomes. The most surprising results we observed were with the woolly genotype which negatively influenced larval beetle weight gain and feeding time. We observed that *L. decemlineata* larvae prefer to feed on leaves with very low density of trichomes. We observed that larval *L. decemlineata* had difficulty finding a suitable feeding site on the woolly mutant leaf which contains a very high density of non-glandular trichomes (personal observation). These results indicate that non-glandular trichomes although harmless or perhaps even beneficial for caterpillar growth, were detrimental to beetle feeding and growth.

We did not find that trichomes impacted oviposition by either insect species in the genotypes we tested. Levin (1973) and others have reported that trichomes influence insect oviposition in a wide range of insects and that trichome length on the leaf surface is often negatively correlated with the number of eggs laid. But in our study, using a choice-test, *H. zea* and CPB adults did not exhibit ovipositional preference for any of the genotypes tested. While we cannot conclude that trichomes have no effect on oviposition for these insects, at least for these genotypes there was not sufficient variation in trichome type or density to uncover differences in ovipositional preference.
Trichome density is often inducible by insect herbivory or by plant hormones (Kessler et al. 2011; Traw and Dawson 2002b). Trichome density is regulated by jasmonates, brassinosteroids and ethylene in tomato (Boughton et al. 2005; Campos et al. 2009). In fact, induction of glandular trichomes by herbivory, wounding, or MeJA treatment in the parent generation persists in the offspring of the parents (Rasmann et al. 2012). In this study, both glandular and non-glandular types of trichomes were up-regulated by MeJA, although the specific trichome type was not induced by hormone treatment. When plants were treated with MeJA, *H. zeae* growth was significantly reduced on treated plants of all genotypes. The growth of *L. decemlineata* was not affected by MeJA treatment in the *hl*, AC or RU genotypes, except in the case of the *woolly* mutant where the non-glandular trichome length was significantly increased by MeJA treatment. The increase in the length of non-glandular trichomes may further impair larval feeding. These results provide further support for the role of non-glandular trichomes in resistance to *L. decemlineata*.

Trichomes are initiated in the epidermis of developing leaves; previous studies on *Arabidopsis* have revealed several key regulators that participate in trichome formation and induction (neeed references). The mechanisms trichome formation in tomato is less clear. Previous study on *hl* mutant showed that the Hl gene plays an important role in the synthesis of new cell wall material in developing trichomes. Cloning of the gene is needed to understand the precise function of Hl in trichome development (Kang et al. 2010b). The *woolly* (*Wo*) gene is responsible for trichome formation and regulates SlCyB2 gene expression that participates in trichome formation. Suppression of *Wo* and *SlCyB2* using RNAi on the mutant (LA3186) produced low densities trichomes the leaf surface (Chen et al. 2008). In our study, we used a
different *woolly* mutant (LA0258) from the previous study and our results showed the SlCyB2 gene was also highly expressed in *woolly* trichomes.

While the induction of high densities of trichomes may impact herbivores, the secondary metabolites produced by glandular trichomes may also negatively impact herbivore feeding and growth. Transcriptional co-regulation is an important hallmark of genes involved in secondary metabolite pathway and our results confirmed that there is high degree of association between monoterpene and sesquiterpene content and gene expression (i.e., *MTS1* (=*TPS5*) and *SST1* (=*TPS9*) ). In addition to terpenes, glandular trichomes contained other defenses including anti-nutritive proteins and phenolics, thus it is not surprising that tomato glandular trichomes have been implicated in resistance to a variety of herbivore species including caterpillars and aphids (Chen et al. 2008; Kang et al. 2010b). For instance, trichomes of many *Solanum* species accumulate significant levels of polyphenol oxidase and play roles in the oxidation of phenolics that may defend against insects and pathogens (Thipyapong et al. 1997). Proteinase inhibitors are another class of defensive proteins in tomato and can accumulate in many plant tissues which affect insect growth (Kang et al. 2010a; Kang et al. 2010b; Ryan 1990). HPL is also associated with plant development and defense. Our results confirmed that *PIN2*, *PPOF* and *HPL* are highly expressed in glandular trichomes. High expression of these proteins in the trichomes may reduce the nutritional quality of dietary protein available to *H. zea* (Felton and Duffey 1992), but not that available to CPB (Felton et al. 1992) due to differences in the physico-chemical environment of their digestive systems (Johnson and Felton 1996; Zhu-Salzman et al. 2008). Moreover, the expression of the *PIN2* gene in the trichomes is induced by *H. zea* feeding indicating that herbivory may impact both the density of trichomes as well as their chemical composition.
Trichomes of the cultivated tomato are an important source of constitutive and inducible resistance to insect herbivores and offer potential for exploitation in plant breeding programs. Novel secondary metabolites are present in the glandular trichomes of wild species of tomato and could be used for sources of host resistance been shown to produce numerous arthropod defensive compounds (Karban and Baldwin 1997; Li et al. 2004). Our results with *H. zea* provide further substantiation that glandular trichomes play a role in resistance; however, our study provides several important caveats for development of host plant resistance programs. Although, *H. zea* growth was compromised on the *hl* mutant, the growth of *L. decemlineata* was not. The use of non-glandular trichomes may be useful in resistance programs to *L. decemlineata*, but could inadvertently enhance susceptibility to some insects such as *H. zea*. Thus our findings underscore the necessity to clarify the roles of specific trichomes to different herbivorous insect species before embarking on a comprehensive plant breeding program.

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Reference
Agrawal AA (1999) Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80: 1713-1723


Kang JH, Shi F, Jones AD, Marks MD, Howe GA (2010b) Distortion of trichome morphology
by the hairless mutation of tomato affects leaf surface chemistry. Journal of Experimental Botany 61: 1053-1064
spectrometry screening reveals widespread diversity in trichome specialized metabolites of tomato chromosomal substitution lines. Plant Journal 62: 391-403


Stratmann JW, Ryan CA (1997) Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors. Proceedings of the National Academy of Sciences 94: 11085-11089


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Fig. 1. Morphology of trichomes on mutant and wild type leaf and stem, and induction by methyl jasmonate (MeJA): (a-c) woolly mutant trichome on leaf, stem and MeJA induction (d-f) Wild type Rutgers (RU) trichomes on leaves and stems, (g-i) hairless (hl) mutant trichome on leaves and stems and induction by MeJA, (j-l) wild type Alisa Craig (AC) trichomes

Fig. 2. Trichome morphology on leaves in wild type and hl, woolly mutant plants under scanning electron microscope: a) woolly mutant with type I and type III trichomes b) hl mutant showing fewer trichomes, mostly type VI trichomes, and some distorted type I trichomes. c) wild type RU leaf with type I, IV and VI trichomes. d) wild type AC leaf with type I, IV and VI trichomes

Fig. 3. Trichome density on hl, woolly mutant and wild type plants and induction by MeJA. a) Density of non-glandular trichome on hl, woolly mutant and wild type leaves, induction by MeJA. b) Density of glandular trichome on hl, woolly mutant and wild type leaves and induction by MeJA. c) Trichome length induced by MeJA. Data show trichome density as the mean (±SE) trichome number of 20 replicate leaves of 4th leaf, trichome length is the mean (±SE) mm of 15 non-glandular trichomes on different leaves. Means followed by different letters are significantly different at P= 0.05).

Fig. 4. Representative total ion chromatograms showing MTBE extractions of leaf trichomes from a) Rutgers (RU; wild type for woolly), b) woolly (non-glandular trichomes), c) hl (glandular trichomes). Compounds are: 1) α-pinene, 2) unknown monoterpene, 3) cis-3-hexenyl acetate, 4) β-phellandrene, 5) nonyl acetate (external standard).

Fig. 5. Comparison of monoterpenes and sesquiterpenes levels in mutant and wild type leaves and stems. a) Monoterpenes in stem and stem b) Sesquiterpenes in stem and leaf c) Leaf dip method for comparison monoterpenes and sesquiterpenes. Each data point represents the mean±SE of three replicates. Means followed by different letters are significantly different at P= 0.05).

Fig. 6. Relative gene expression in different types of trichomes in hl, woolly and wild type. Trichomes were extracted from 30 plant leaves, relative expression is calculated to wild type RU leaf RNA. Bars indicates standard error of three technical replicates. PIN2 proteinase inhibitor 2 gene, MTS1 (=TPS5) monosesquiterpene synthase 1 gene, SSI1 (=TPS9) Sesquiterpene synthase 1, PPO polyphenol oxidase gene, SlCycB2, B type cyclin gene. HPL, Hydroperoxide lyase gene. Means followed by different letters are significantly different at P= 0.05).

Fig. 7. hl and woolly mutant affect Helicoverpa zea and Colorado potato beetle growth and feeding time. No choice bioassay were performed by placing first instar H. zea and CPB for 4 days bioassay. a) hl, woolly mutants and wild type influence H. zea growth. b) hl, woolly mutants and wild type influence CPB growth. c) Feeding time influenced by different mutants compared to wild type plants. Data represents mean±SE of larval weight (n=30); data of feeding time represents mean±SE of larvae (n=15) feeding on leaves. Means followed by different letters are significantly different at P= 0.05).

Fig. 8. H. zea feeding induced relative expression of proteinase inhibitor 2 (PIN2) in mutant and wild type plants. woolly is the woolly (hairy) mutant, hl is the hairless mutant, RU and AC are the wild type of woolly and hl mutant. Relative expression of PIN2, Means followed by different letters are significantly different at P= 0.05).
Fig. S1. Oviposition choice on different mutants. Not significantly different among the mutants. F= 0.03, P= 0.993 for *H. zea*; F=0.09, P=0.967 for CPB (n=8)

Fig. S2. Oviposition of *H. zea* and CPB in woolly mutant. a) *H. zea* egg on woolly leaf surface with non-glandular trichomes. b) *H. zea* eggs on woolly stem with both glandular and glandular trichomes. c) CPB lay eggs on underside leaf of woolly mutant
Fig. 1.
Fig. 2.
Fig. 3.
Fig.4.
Fig. 5
Fig. 6
Fig. 7.
Fig. 8.
Fig. S1.
Fig. S2.
Table S 1. Monoterpene in mutant and wild type trichomes (ng/fresh weight g)

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<td>189.5±26.9</td>
<td>231.6±16.1</td>
<td>12137.4±53.7</td>
<td>307.7±14.1</td>
<td>28.0±8.5</td>
<td>37958.8±338.6</td>
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Table S 2. Sesquiterpenes in mutant and wild type trichomes (ng/fresh weight g)

<table>
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<tr>
<th></th>
<th>z-3-hexenyl butyrate</th>
<th>e-2-hexenyl butyrate</th>
<th>z-jasmone</th>
<th>caryophyllene</th>
<th>α-humulene</th>
<th>β-farnesene</th>
<th>nerolidol</th>
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<tr>
<td>hl stem</td>
<td>Nd</td>
<td>76.17±10.39</td>
<td>18.22±5.68</td>
<td>33.62±2.93</td>
<td>nd</td>
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<tr>
<td>hl leaf</td>
<td>643.07±78.8</td>
<td>2318.10±323.33</td>
<td>704.62±88.77</td>
<td>902.62±152.55</td>
<td>282.14±29.67</td>
<td>354.61±39.43</td>
<td>206.77±25.73</td>
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<tr>
<td>AC stem</td>
<td>17.2±5.12</td>
<td>67.43±18.07</td>
<td>35.69±13.81</td>
<td>64.98±16.35</td>
<td>nd</td>
<td>11.75±2.53</td>
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<tr>
<td>AC leaf</td>
<td>234.87±67.3</td>
<td>786.18±232.68</td>
<td>340.80±69.85</td>
<td>582.34±168.57</td>
<td>144.13±27.95</td>
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<td>woolly stem</td>
<td>nd</td>
<td>53.23±5.19</td>
<td>15.25±6.31</td>
<td>23.70±14.32</td>
<td>nd</td>
<td>6.47±3.49</td>
<td>nd</td>
</tr>
<tr>
<td>Woolly leaf</td>
<td>nd</td>
<td>nd</td>
<td>31.47±10.23</td>
<td>81.92±18.59</td>
<td>35.21±3.73</td>
<td>21.95±10.23</td>
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<td>RU stem</td>
<td>49.54±4.98</td>
<td>128.91±10.89</td>
<td>54.29±7.05</td>
<td>40.92±3.68</td>
<td>24.46±4.78</td>
<td>21.18±4.50</td>
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<td>RU leaf</td>
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Chapter IV
Roles of Ethylene and Jasmonic Acid in Systemic Induced Defense in Tomato (Solanum lycopersicum) against Helicoverpa zea

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Abstract

The plant defense-signaling is mediated by the synthesis, movement, and perception of jasmonate (JA), and through the interaction with other plant hormones and messengers. To characterize the interaction of ethylene and JA on induced defenses in tomato, we used the never ripe (Nr) mutant, which contains a partial block in ethylene perception and the defenseless (def1) mutant, which is deficient in JA biosynthesis. The defense gene proteinase inhibitor (PIN2) was used as marker gene to compare plant responses. The Nr mutant showed a normal wounding response with PIN2 induction, whereas the def1 mutant did not. As expected, methyl JA (MeJA) treatment restored the normal wound response in the def1 mutant. Exogenous application of MeJA increased resistance to Helicoverpa zea, induced defense gene expression, and increased glandular trichome density on systemic leaves. Exogenous application of ethephon, which penetrates tissues and decomposes to ethylene, resulted in increased H. zea growth and interfered with the wounding response. Ethephon treatment increased salicylic acid (SA) in the systemic leaves. These results showed that while JA plays the main role in systemic induced defense, ethylene acted antagonistically to negatively regulate systemic defense in tomato to H. zea.

Key words: methyl jasmonate, ethephon, induced systemic defense, insect herbivore, trichomes, proteinase inhibitor, hormones, jasmonic acid, salicylic acid, and elicitors
**Abbreviations:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Nr</td>
<td>Never ripe mutant</td>
</tr>
<tr>
<td>def1</td>
<td>Defenseless 1 mutant</td>
</tr>
<tr>
<td>RU</td>
<td>Rutgers wild type plants of Nr mutant</td>
</tr>
<tr>
<td>CM</td>
<td>Castlemart, wild type plants of def1 mutant</td>
</tr>
<tr>
<td>MeJA</td>
<td>methyl jasmonate</td>
</tr>
<tr>
<td>PIN2</td>
<td>protease inhibitor 2</td>
</tr>
<tr>
<td>ERF1</td>
<td>ethylene response factor</td>
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<td>PRI1</td>
<td>pathogenesis related gene</td>
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<td>PPOF</td>
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<td>ethylene</td>
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<tr>
<td>SA</td>
<td>salicylic acid</td>
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Introduction

Biotic and abiotic stresses negatively impact plant health and fitness. Plants respond to the biotic stress of insect herbivores and pathogens by inducing systemic resistance pathways. Plant hormones play important role in regulating this defensive process (Erb et al. 2012; Howe and Jander 2008). Among them, jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) have been intensively investigated in terms of plant defense against pathogen infection or insect herbivory (Abe et al. 2011; Fan et al. 2009; Leon-Reyes et al. 2009). Other plant hormones, such as brassinosteroids (BA), gibberellins (GAs) and auxins have also been reported as part of the defense-signaling network (Navarro 2006; Yasuda et al. 2003; Zsogon et al. 2008).

JA is a naturally occurring phytohormone that occurs ubiquitously in plant species and functions in regulation of seed germination, root growth, pollen development and fruit ripening (Zhang et al. 2011). In addition to its role in plant growth development, JA and its precursors perform an important function in regulating host defensive responses. The regulation of plant defense by JA has been studied by observing the gene expression patterns following exogenous application of jasmonates or through the use of loss of function or gain of function mutants (Browse 2009; Howe 2004). Proteinase inhibitors (PINs) and polyphenol oxidases (PPO) are two of the best studied groups of JA-regulated defense proteins in vegetative tissues. Wound-induced proteinase inhibitors have been shown to enhance the plant’s resistance to insects by inhibiting the proteolytic enzymes of the attacking insects (Koiwa et al. 1998). In some instances, JA partially regulates trichome production, which functions in plant defense (Halitschke et al. 2008; Li et al. 2004; Traw and Bergelson 2003; Tian et al. 2012b). In tomato, application of methyl jasmonate (MeJA) increases densities of glandular trichomes on new leaves (Boughton et
The inducibility of trichome density is ecologically significant because increased trichome density can negatively influence herbivore populations (Agrawal 1999; Horgan et al. 2009; Kessler et al. 2011).

In addition to jasmonic acid, additional plant hormones are known to regulate plant defenses. Ethylene was the first identified gaseous hormone and it is involved in many plant physiological process throughout plant development including, seed germination, flowering, fruit ripening, organ senescence and response to stress (Zhang et al. 2010). Previous study showed that the key components of ethylene biosynthesis, 1-amino-cyclopropane - 1carboxylic acid synthesis (ACS) and 1-aminocyclopropane-carboxylic acid oxidase (ACO) are critical for ethylene biosynthesis (An et al. 2006; Yang and Hoffman 1984). Regulation of the ethylene pathway is important in mediating plant development and stress response (Diaz et al. 2002; Mantelin 2009; Zhang et al. 2011). Ethylene accumulation during pathogen infection up-regulates the expression of many genes in plants (Diaz et al. 2002). Ethylene response factors (ERFs), which represent the second largest transcription factor family in plants, have been shown to integrate signals from different plant hormone pathways and play important roles in stress response (Onate-Sanchez et al. 2007).

Hormone cross talk is a process where different hormone signaling pathways interact antagonistically or synergistically to regulate responses to complex and changing environments (Verhage et al. 2010). There is ample evidence for cross talk among SA, JA and ET signaling pathways in plant immunity (Leon-Reyes et al. 2009). Cross talk likely minimizes fitness costs and creates a flexible signaling network that allows the plant to fine-tune its defense response to the appropriate invaders (Bostock 2005; Thaler et al. 2012;Pieterse and Dicke 2007). In some instances, JA may collaborate with the SA response pathway to trigger an indirect defense
against an insect herbivore as observed in Arabidopsis (van Poecke and Dicke 2002). Walling (2000) found that phloem-feeding insects may induce both SA and JA dependent pathways. Simultaneous expression of SA and JA pathways was observed in the Mi-gene mediated resistance in tomato during aphid feeding (Martinez de Ilarduya et al. 2003). While in tobacco, it was observed that there was an inverse relationship between endogenous concentrations of SA and JA (Felton et al. 1999). Exogenous application of SA has been shown to inhibit both JA synthesis and downstream defenses in tomato (Chandok et al. 2004; Thaler et al. 2010). Moreover it was shown that ET enhanced the plant response to SA, resulting in the expression of the SA-responsive marker gene pathogenesis-related-1 (PR-1) (Leon-Reyes et al. 2009). ET can synergistically or antagonistically interact with JA in the regulation of plant development and stress responses (O'Donnell et al. 1996; Zhao 2003). Many defense-related genes such as proteinase inhibitors are regulated via a signaling pathway that requires both ET and JA (Adie et al. 2007a; Pieterse et al. 1998). In maize, the inhibition of ET biosynthesis or perception increased herbivore performance and damage in treated plants (Harfouche et al. 2006). However ethylene acts antagonistically to suppress JA induced expression of nicotine biosynthesis in tobacco (Rojo et al. 1999; Shoji et al. 2000). Exogenous ethylene application on Arabidopsis increased the growth rate of the Egyptian cotton worm (Mantelin 2009; Stotz et al. 2000). These studies on the interaction of plant hormones frequently show different impacts on plant defense, but some of the differences may be attributed to varied methodologies, treatments, etc. Furthermore, the interaction of plant hormones in defense depends on the specific plant-herbivore system studied.

Tomato has been a primary model system for elucidating induced defenses and their signaling pathways against herbivores, yet most of the research has focused on signaling and
rapidly-induced defenses in leaves (Melotto et al. 2008). Considerably less study has focused on systemic induction and the induction of defenses that require days to weeks to generations for expression (Rasmann et al. 2011; Tian et al. 2012a). In this study, we used tomato mutants and application of hormones to dissect the interactions of JA and ethylene that regulate plant systemic defense to a chewing herbivore, Helicoverpa zea. We used the tomato never ripe (Nr) mutant, which is impaired in ethylene-mediated plant response and the defl mutant which is deficient in JA biosynthesis to study the hormone interactions. We used a combination of insect bioassay, defense gene expression, and plant hormone analysis to identify the key interactions between JA and ethylene.

**Materials and Methods**

**Plant materials and insects**

Tomato (*Lycopersicon esculentum*) seeds of the defl mutant and wild type Castlemart (CM) were kindly provided by Dr. Gregg Howe (Michigan State University). Seeds for the Nr mutant (accession number LA3001) and the wild type Rutgers (RU, accession number LA1090) were obtained from the Tomato Genetics Resource Center (University of California, Davis). All the plants were grown in Metromix 400 potting mix (Griffin Greenhouse & Nursery Supplies Tewksbury, MA) in a greenhouse at Pennsylvania State University, University Park, PA, USA). The greenhouse was maintained on a 16-h photoperiod (Peiffer and Felton 2005). Tomato fruitworm (*Helicoverpa zea*) eggs were purchased from BioServ (Frenchtown, NJ) and were reared on a wheat germ and casein-based artificial diet (Chippendale 1970) with ingredients from Bioserv.

**Plant treatments**
To compare the effect of exogenous hormones on plant defense, tomato plants were sprayed with 2.5 mM methyl jasmonate (MeJA) in 0.8% ethanol or 3 mM ethephon (2-chloroethylphosphonic acid, CEPA, Sigma, St. Louis, MO, USA) at the four-leaf-stage. The treated plants were allowed to grow in the greenhouse for another two weeks after treatment.

Two weeks after the spray treatments, the plant height was measured to compare how the exogenous hormones affected plant growth. Two leaf discs from the top fully expanded leaf were cut from each side of the mid-vein. Glandular trichome numbers were counted under a light microscope on each leaf disc. The density of trichomes was calculated as the number per disc/cm².

To compare how plant hormones regulate the wounding response, two weeks after treatment, the newly emerged leaves on treated tomato plants were mechanically wounded by punching two ¼ inch diameter holes along the mid-vein of the terminal leaflet. Twenty-four hours after the wounding treatment, the wounded leaf was used for RNA extraction and gene expression assay.

To examine the effect of hormone treatment on seedling growth, we surface sterilized seeds and allowed them to germinate on agar plates with 100µM MeJA or 100µg/L ethephon in Murashige and Skoog (MS) medium (Sigma, St. Louis, USA). One week later, we compared seedling growth by measuring the hypocotyl and root length. Also we collected the seedlings to compare the effect of hormone treatment on defense gene expression.

**Insect Bioassays**

To determine if the hormones directly affected fruitworm growth, we used an artificial diet bioassay. Based on diet weight, 100µg/g MeJA or 100µg/g ethephon was added on the diet
surface. After twenty minutes of drying, one neonate larva was placed in each cup. A total of thirty larvae were tested per treatment.

Detached leaf bioassays were conducted to compare how different plant treatments affected insect growth. Newly hatched *H. zea* larvae were reared on artificial diet for one day before transfer to the bioassay cups. A detached newly emerged leaf from plants was used for the bioassay. The treated leaves were put in a standard 1 oz. cup (BioServ, Frenchtown, NJ) with 2% agar on the bottom to maintain moisture. Insects were kept at 27°C, with a 16:8 L:D photoperiod in an incubator. Thirty insects were used for each treatment. After five days, larval weights were recorded to the nearest 0.1 mg.

A choice test with MS medium grown seedling was conducted to compare how different treatments affect larval choice. The control and two treated seedlings were placed in the edge of a 150x15mm petri-dish and fifteen first instar larvae were placed in the center. All experiments were repeated three times. After 24 hours, we compared the number of caterpillars on each seedling.

**RNA extraction and quantification analysis**

To extract RNA, 100 mg leaf sample for each replicate was homogenized in liquid nitrogen and total RNA was purified with a RNaseasy Plus Mini-kit (Qiagen, Valencia, CA, USA). High Capacity Transcription kit (Applied Biosystems, Foster City, CA) was used for cDNA synthesis. All quantitative RT-PCR (qRT-PCR) reactions used Power SYBR Green PCR Master Mix and ran on 7500 Fast Real-Time PCR system (Applied Biosystems) following standard protocols (10 min at 95°C, with 40 cycles of 15s at 95°C and 60s at 60°C). The ubiquitin gene was used as the housekeeping gene to normalize $2^{-\Delta\Delta C(T)}$ (Rotenberg et al. 2006).
**Plant hormone analysis**

Plant hormone levels were determined two weeks after JA or ethephon treatment. To measure hormones, 100 mg of newly emerged leaves were collected into FastPrep® tubes (Qbiogene, Carlsbad, CA) containing 1 g of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ), and frozen at -80°C until processing. The new emerged leaf would not have been directly exposed to the hormone treatments.

To extract and detect JA and SA, we used a previously described method (Tooker and De Moraes, 2005) which was modified slightly from (Schmelz et al. 2003). Briefly, we derivatised the carboxylic acid to its methyl ester, which was isolated using vapor phase extraction and analyzed by GC-MS with isobutane chemical ionization using selected-ion monitoring.

**Statistical Analysis**

Data were analyzed using general linear models for analysis of variance (ANOVA) where appropriate with post-hoc comparison of means using the Fisher-LSD means separation (MiniTab Software, State College, PA).

**Results**

**Hormone application with ethephon and MeJA affect tomato growth**

To examine how MeJA and ethephon affect plant growth, plant height was measured two weeks after treatments. The *def1* and *Nr* mutant without hormone treatment grew normally during this period and did not show any growth reduction compared to their respective wild type plants. MeJA/ethephon treatments exhibited significant changes on tomato growth. Compared to the untreated plants, plant height in the *Nr* mutant was reduced 12.5% by MeJA treatment and 31.0% by the ethephon treatment. While *def1* mutant plant height was reduced 34.5% and 69.2
% by each treatment respectively. Although \textit{Nr} mutant displays reduced ethylene sensitivity, the ethephon treatment still reduced growth. Both wild type plants showed significantly reduced plant growth after the treatment. After ethephon treatment, the height of RU and CM plants were reduced by 54.1\% and 52.3\% respectively. The MeJA treatment reduced the height by 22.6\% and 26.1\% on both RU and CM plants (Fig. 1A, $F=97.48$, $p<0.01$).

When MeJA and ethephon were both added to MS medium plates at same time, the seedling growth was significantly affected by the treatment. Compared to control seedlings, both MeJA and ethephon significantly reduced hypocotyls length and root growth (Fig. 1 B; $F=28.73$, $p<0.001$ and C; $F=7.01$, $p<0.001$).

\textbf{Trichome production regulated by hormone application}

A striking reduction in type VI glandular trichome density was observed on JA deficient \textit{def1} mutant compared to the wild type CM trichome number (Fig. 2A; $F=27.18$, $p<0.01$). The trichome numbers on the \textit{def1} mutant were 40.9\% less than on CM. While the trichome numbers on the \textit{Nr} mutant did not show significant differences compared to wild type Rutgers (RU) plants (Fig. 2B; $F=0.15$, $p=0.696$).

The MeJA and ethephon showed different effects on trichome production. The trichome density on both mutants and wild type plants was significantly increased by MeJA treatment. The \textit{def1} and CM showed a 67.5\% and 71.4\% increase respectively. The trichome density on \textit{Nr} and RU was increased by 81.6\% and 71.5\% compared to the untreated control. Conversely the trichome density on ET-treated plants was decreased from 6.5\%-27\%, but with no significant reduction compared to the untreated mutants and wild type plants (Fig 2 A, B.). These results are consistent with a previous study where MeJA induced trichomes on new growth leaves and confirms that JA plays important role in trichome formation (Boughton et al. 2005). The effects
of ethylene on trichome production have shown different results from previous studies, but may be due to differences in application time, concentration or different hosts (Gibson 2003; Plett et al. 2009).

**Hormone application and systemic resistance against *H. zea***

While various studies have shown that elicitors such as JA and ET regulate plant defense to insect feeding (Adie et al. 2007a; Ankala et al. 2009), we determined if the elicitors had any direct effects on the insect. MeJA or ethephon in diet did not directly affect larval growth rate over a five-day feeding period (Fig. 3A; \( F=0.23, p=0.799 \)).

We then tested the growth of larvae on the ethylene and JA mutants. The bioassays with leaves from *Nr* mutant and RU wild type plants showed that *H. zea* growth was not significantly different between the two genotypes (Fig. 3B; \( F=9.42, p<0.01 \)). However, the growth of *H. zea* significantly increased on the *def1* mutant compared to its wild type CM (Fig. 3B). To assess how JA and ET treatments of plants affected insect growth, the new growth leaves were removed for bioassay. The results showed that treatment of plants with ethephon significantly increased caterpillar growth on both mutants and wild type plants compared to control plants (Fig. 3B). Because a small amount of ethanol is added to dissolve MeJA, we also used an ethanol control treatment; nevertheless MeJA significantly reduced the caterpillar growth compared with the ethanol treatment in all the mutants and wild type plants (Fig. 3C; \( F=17.91, p<0.01 \)). The choice test with MS medium grown seedlings showed ethephon treatment seedlings were more attractive to first instar larvae than MeJA and control treatment (Fig. 3D; \( F=29.88, p<0.01 \)).

**MeJA and ethephon treatment effects on plant gene expression**
Proteinase inhibitors (e.g., PIN2) are known to act as defensive proteins against insects by affecting their larval nutrition (Ryan et al. 1986; Wasternack et al. 2006). The PIN2 defense gene is mainly regulated by JA, which syntheses via the octadecanoid pathway (Howe 2004). Our results showed there were no difference in the constitutive expression of PIN2 between the Nr mutant and its wild type RU. While in the def1 PIN2 expression was significantly lower compared to its wild type CM leaves as expected (Fig. 4A; F=8.19, p<0.01).

To investigate the roles of ET and JA in defense gene expression, we compared PIN2 expression on systemic leaves after ethephon or MeJA treatment. PIN2 expression on Nr mutant was not significantly reduced after the ethephon treatment compared with untreated plants; however the wild type plants showed significant reduction after the ethephon treatment. Ethephon treatment on CM plants also significantly reduced PIN2 expression, but in the case of the def1 mutant, the steady state levels of PIN2 are already very low and were not further reduced by ethephon treatment. In general, the ethephon significantly reduced PIN2 expression on systemic leaves of wild type genotypes. Compared to control ethanol treatments, MeJA treatments significantly induced PIN2 expression in both mutants and wild types. After MeJA spray, the PIN2 expression for the def1 mutant was not significantly different from the treated wild type CM plants (Fig. 4B, F=0.15, p=0.929), indicating that this mutant is still responsive to JA as expected.

After 7 days growth on MS medium, whole seedlings were collected for analysis of PIN2 gene expression. The results showed the same trend of PIN2 expression on MS medium as the treatment with ethephon significantly reduced PIN2 expression (Fig. 5A; F=6.49, p<0.01). The PIN2 expression on MS medium with MeJA treatment was significantly induced compared with the control seedlings. The expression of PIN2 was significantly lower than MeJA alone
treatment when both elicitors were added in the medium, (Fig. 5B, F=9.87, p<0.01). This indicates that as expected, JA plays important role in PIN2 induction; however, ET negatively regulates PIN2 expression through an antagonistic interaction.

In addition to PIN2 expression, we also examined PPOF, ERF1 and PRI1 expression in the MS medium grown seedlings. Polyphenol oxidase (PPOF) plays a defensive role in plant defense against insects (Bi et al. 1997; Felton et al. 1989; Thipyapong and Steffens 1996). The results in this study showed that PPOF expression was also significantly decreased by ethephon treatment, while highly induced by MeJA treatment. Thus ethephon negatively regulated PPOF expression in a manner similar to PIN2 (Fig. 5C, D).

Previous study showed multiple tomato PR proteins and their corresponding mRNAs are regulated by salicylic acid and ethylene treatment (Vankan et al. 1995). The ERF1 gene which play important role in stress response is regulated by ethylene (Nakano et al. 2006; Onate-Sanchez et al. 2007). Our results showed significant induction of ERF1 and PRI1 by ethephon on def1 mutant and wild type seedlings. The Nr mutant, which is semi-dominant ethylene insensitive, showed no significantly induction of ERF1 and PRI1 expression compared to control. The wild type RU showed significantly induction of both genes (Fig. 5E, F, G, H). These results are consistent with a previous study indicating that ethylene and SA positively regulate ERF1 and PRI1 expression (Berrocal-Lobo et al. 2002; Huang et al. 2005). MeJA treatment did not significantly induce either gene, while MeJA and ethephon together produced similar results as ethephon alone. These results confirmed that ERF1 and PRI1 gene are up regulated by ethylene.

**JA but not ethylene is required for PIN2 wound-induced expression**
Because *PIN2* is a well-characterized wounding response gene in tomato, we used this as marker to compare the wounding response in different mutants and after different hormone treatments. We compared the *PIN2* gene expression 24 h after wounding. RNA from untreated leaves of the wild type RU was used as a reference level to calculate the relative *PIN2* expression across other treatments. The *Nr* mutant, which has partially reduced ethylene response, showed significantly greater *PIN2* induction after mechanical wounding compared to the wild type RU plants. The *def1* mutant, which lacks a functional octadecanoid pathway, did not show significant *PIN2* induction upon wounding. Its wild type CM showed a significant wounding response compared to control untreated plants (Fig. 6A; F=23.19, p<0.01).

To investigate the effect of ethephon and MeJA on the systemic wounding response, plants were sprayed as previously described. After two weeks, the 5th leaf on plants was wounded as previous described. Twenty-four hours after mechanical wounding, the *PIN2* expression was compared with RU untreated plants. The results showed after ethephon treatment, the plants still showed a wounding response with induced *PIN2* expression compared to untreated plants. The *def1* mutant had still lower *PIN2* expression compared to CM plants (Fig. 6B; F=9.78, p<0.01). After MeJA spray the plants showed a normal wounding response. The *PIN2* gene expression on both *Nr* and *def1* mutants and wild type plants was significantly increased compared to the untreated plants, but no difference among mutants and wild type plants was observed (Fig. 6C; F=2.10, p<0.179). In this study, the *ERF1* gene did not show any induction upon wounding in both mutants and wild type plants although *def1* mutant showed high *ERF1* expression (Fig. 6D; F=110.92, p<0.01). *PRI* gene was up-regulated by mechanical wounding on *def1* mutant, but no induction was observed on the *Nr* mutant and two wild type plants (Fig. 6F; F=97.65, p<0.01).
Plant hormones in systemic leaves

To understand how the cross interaction of JA and ET affects plant hormones, we quantified SA and JA on the systemic leaves two weeks after treatment. After ethephon treatment, the SA levels in the Nr mutant systemic leaves increased 2.6 times compared to the control plants (Table 2). Wild type RU plants showed a 6.4 fold increase compared to the control. After ethephon treatment, the SA levels in both the def1 mutant and wild type CM plants increased ca. 10-fold compared to control plants. While the MeJA treatment did not significantly increase SA levels in both mutants and wild type systemic leaves. Expectedly, the JA quantity was significantly increased on systemic leaves compared to ethanol treatment after MeJA treatment. The ethanol treatment did not show any effect on JA levels. JA levels in the systemic leaves of the Nr and def1 mutants showed no significant decrease after ethephon treatment; however, in the RU plants, JA was reduced after ethephon treatment compared to control plants. However, JA in CM plants was not reduced by ethephon treatment (Table 2).

Discussion

The plant hormones SA, JA and ET are known to play the important signaling roles in local and systemic plant defense insect herbivores (Howe 2004; Onkokesung et al. 2010). Among them, JA signaling plays a prominent role in promoting plant defense responses to many herbivores (Bodenhausen and Reymond 2007; Cooper and Goggin 2005; McConn et al. 1997). Other hormones such as SA and ET may interact with JA in regulating plant defense (Adie et al. 2007a; Beckers and Spoel 2006; Campos et al. 2009; Wasternack et al. 2006). Both positive and negative effects of ET on JA regulated responses have been observed (O'Donnell et al. 1996; Stotz et al. 2000).
Our results confirm the role of JA in defense against the caterpillar *H. zea*. Caterpillar growth was significantly increased on the *def1* mutant compared with the wild type plants. Pretreatment of tomato plants with MeJA significantly decreased *H. zea* growth on both mutants and wild type plants. The MeJA treatment rescued *def1* mutant resulting in increased resistance to the insect herbivore. Larval growth on the *Nr* mutant was not affected compared to the wild type plants, but pretreatment with ethephon compromised resistance to *H. zea* in both *Nr* and wild type plants. This antagonistic interaction has also been reported in other plants such as a previous study showing that pretreatment of Arabidopsis with ethephon, elevated susceptibility to generalist insects including the Egyptian cotton worm (Stotz et al. 2000). Ethylene negatively regulated local expression of lectin GS-II in *Griffonia simplicifolia*, which inhibited insect growth and development (Zhu-Salzman et al. 1998). Ethylene also negatively regulates JA-induced nicotine biosynthesis and reduced direct plant defenses (Kahl et al. 2000). In contrast maize blocking ethylene synthesis and perception in maize resulted in plants more susceptible to caterpillar feeding (Harfouche et al. 2006; Ankala et al. 2009). These studies suggest that the role and outcome of ET in defense may depend on the specific plant-herbivore system being studied.

Glandular trichomes are an important component of the defended phenotype of many plants. Trichome density is regulated by jasmonates, brassinosteroids and ethylene (Boughton et al. 2005; Campos et al. 2009; Tian et al. 2012). JA plays a positive role in glandular trichome induction in different plant species (Peiffer et al. 2009; Rasmann et al. 2012; Traw and Bergelson 2003; van Schie et al. 2007). In this study, the *def1* mutant with JA deficiency showed significantly trichome reduction compared to its wild type CM plants; while the *Nr* mutant did not show any trichome reduction compared to wild type RU plants although the ethylene perception was partially blocked. JA significantly induced glandular trichomes on systemic
leaves, while ethylene did not affect tomato glandular trichome density. There are different reports on ET function in regulating trichome formation; for example in Arabidopsis ET inhibits trichome formation on stems and leaves (Gibson 2003), while another study showed ET positively regulated plant cell division and increased trichome initiation and development (Kazama et al. 2004). Exogenous application of ET has been found to increase the branch number in cucumber trichomes and increased ethylene synthesis has been correlated with trichome branching (Plett et al. 2009b). Differences in these studies may be due to specific plant species or differences in application rates and timing. Ethylene may regulate non-glandular trichome branching, but we did not examine its possible effect on these trichomes.

Plants respond to wounding or insect herbivory by activating a series of wound-responsive defensive genes (Baldwin et al. 2001; Bergey et al. 1999; Chen et al. 2004; Heinrich et al. 2011). Jasmonates are recognized as essential signals in this process (Gfeller et al. 2010). As expected, we observed lower expression of PIN2 in the def1 mutant compared to CM plants. Wounding did not induce significant PIN2 expression in the def1 mutant compared to wild type CM plants. Nr, which is an ethylene insensitive mutant, showed normal constitutive and wound-induced PIN2 expression as the wild type RU plants. This confirmed that JA, but not ET is required for the wounding response. The wounding response in systemic leaves was also affected by ethephon and MeJA treatment. Not surprisingly, the MeJA treatment restored the def1 mutant to a normal wounding response. The ethephon treatment decreased the wound-inducible PIN2 gene expression in systemic leaves. Similar study also found ethylene treatment reduced proteinase inhibitor 1(PIN1) gene expression in tomato (Diaz et al. 2002).

However a synergistic interaction of ET and JA in the regulation of proteinase inhibitor genes has been shown in some instances (Adie et al. 2007b; O’donnell et al. 1996). Our study
showed MeJA application induced $PIN2$ gene expression in systemic leaves, while ethephon treatment decreased $PIN2$ gene expression in these leaves. The assays conducted with seedlings grown on MS medium showed the same trend of $PIN2$ gene expression as the spray treatment. Polyphenol oxidase F ($PPOF$) was similarly up-regulated by MeJA treatment and down-regulated by ethephon treatment. Thus our results contradict some of these previous reports indicating that wound-induced $PIN2$ gene expression depends on both JA and ET (O’donnell et al. 1996). In those experiments, specific inhibitors or elicitors, which regulate ET on plants, were applied to leaves and gene expression on local leaves was quantified after wounding, whereas in our study we focused on $PIN2$ expression in systemic leaves. Also in some cases the methods were different: in our study we used 3 mM ethephon spray on the plants, whereas some previous studies incubated plants in gas tight chambers with ethylene for a short time before wounding plants. The method of gene expression analysis may also have affected the results; in our study, we used quantitative RT-PCR to quantify the gene expression change upon treatment rather than northern blots or semi-quantitative RT-PCR.

The ethylene response factor 1 ($ERF1$) acts as a positive regulator of JA and ET signaling and has been shown to play an important role in mediating defense responses in Arabidopsis (Lorenzo et al. 2003). The expressions of $ERF1$ and the SA-regulated $PRI$ genes in the MS media-grown seedlings were not significantly induced by MeJA treatment, but were significantly induced by ethephon treatment. We did not find $ERF1$ to be induced by our method of mechanical wounding in both tested plants. The $def1$ mutant showed high constitutive $ERF1$ expression compared with wild type plants. Over all in this study, the ethephon treatment induced $ERF1$ expression and decreased the $PIN2$ wounding response. This reduction is consistent with a previous report that $ERF1$ represses wound-responsive $PDF1.2$ gene in
Arabidopsis (Lorenzo et al. 2003). PRI transcripts are regulated by high levels of SA and pathogen attack in tomato plants (Tornero et al. 1997), but the effect of wounding on PRI expression was not previously reported. In this study, the Nr mutant and wild type plants did not show PRI induction by mechanical wounding, while this gene was induced by wounding in the def1 mutant. Because the def1 mutant is deficient of JA biosynthesis, the lack of JA as a negative SA regulator may explain the induction of PRI. This current study is consistent with previous studies of ERF1 and PRI genes that were shown to be up regulated by ethylene response (Cole et al. 2004; Lorenzo et al. 2003).

Significant progress has been made in identifying the key components and understanding the interactive roles of SA, JA and ET in plant responses (Abe et al. 2011; Kniskern et al. 2007; Leon-Reyes et al. 2009). In this study, we showed that ethephon treatments significantly increased SA levels in systemic leaves and decreased JA levels in systemic leaves. Significant ERF1 and PRI gene induction was observed when SA levels were increased by ethephon treatment. Our results confirm that the JA pathway plays the main role in plant defense against H. zea. JA positively regulated PIN2 gene expression and glandular trichome density, but ET positively regulated SA biosynthesis and response with the induction of ERF1 and PRI gene expression. Whereas ET frequently interacts synergistically with JA to mediate insect resistance in some plant species, we found that ET inhibited induced resistance against the tomato fruitworm through decreasing the JA response as observed by decreased PIN2 and PPOF gene expression and insect bioassays.
Table 1. Primers used for real-time PCR assays of relative expression

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene Name/ Accession number</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIN2</td>
<td>Wound inducible proteinase inhibitor 2 / K03291</td>
<td>GGA TTT AGC GGA CTT CCT TCT G ATG CCA AGG CTT GTA CTA GAG AAT G</td>
</tr>
<tr>
<td>PPOF</td>
<td>Polyphenol oxidase /z12837</td>
<td>ACT TTC ACG CGG TAA GGG TT A CTA GTA ACG AGT</td>
</tr>
<tr>
<td>PR1</td>
<td>Pathogenesis related gene1 /NM 001247429</td>
<td>GTC CCC AGC TG T TT G AT TT GG TT G AT T CTA TGA CAT G</td>
</tr>
<tr>
<td>ERF1</td>
<td>Ethylene response factor1 /EU395634</td>
<td>AATGGAATTAGGGTTTTGTTAGGAA GACCAAGGACCCCTCATTGA</td>
</tr>
<tr>
<td>Ubi</td>
<td>Ubiquitin / X58253</td>
<td>GCC AAG ATC CAG GAC AAG GA GCT GCT TTC CGG CGA AA</td>
</tr>
</tbody>
</table>
Table 2: Salicylic acid and JA affected by MeJA and Ethephon
<table>
<thead>
<tr>
<th></th>
<th>Salicylic acid (ng/gm fresh weight)</th>
<th>JA (ng/gm fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ethephon</td>
</tr>
<tr>
<td>Nr</td>
<td>11.33±2.27cd</td>
<td>29.83±7.67b</td>
</tr>
<tr>
<td>RU</td>
<td>12.54±3.92c</td>
<td>73.95±8.14a</td>
</tr>
<tr>
<td>def1</td>
<td>6.32±2.85d</td>
<td>66.97±11.36a</td>
</tr>
<tr>
<td>CM</td>
<td>8.90±1.38d</td>
<td>67.72±13.02a</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

**Fig. 1.** Tomato growth affected by MeJA and ethephon treatment. A.) MeJA and ethephon reduce tomato height  B.) Wild type RU seedling growth by different treatment  C.) Hypocotyl length was affected by different treatment  D.) Root length of different mutant and affected by ethephon, MeJA and combined treatment. Each data point represents the mean±SE of twenty plants. Means followed by different letters are significantly different at P= 0.05.

**Fig. 2.** MeJA and ethephon treatment induce trichome number on systemic leaves. A.) Glandular trichome number on Nr mutant and its wild type leave and affected by ethephon and MeJA treatment. B.) Glandular trichome number on def1 mutant and its wild type CM leaves and affected by ethephon and MeJA. Each data point represents the mean±SE of twenty leaves. Means followed by different letters are significantly different at P= 0.05.

**Fig. 3.** MeJA and Ethephon affect *H. zea* growth and neonates’ choice A.) Artificial diet with MeJA and Ethephon didn’t affect insect growth  B.) Ethephon treated leaves increase *H. zea* growth  C.) MeJA treated leaves reduce *H. zea* growth. Each data point represents the mean±SE of thirty insects. Means followed by different letters are significantly different at P= 0.05.

**Fig. 4.** Chemical elicitors spray affects gene expression A.) *PIN2* expression on leaves was reduced by ethephon treatment B.) *PIN2* expression was highly induced by MeJA spray treatment. Each data point represents the mean±SE of three replicates. Means followed by different letters are significantly different at P= 0.05.

**Fig. 5.** MS medium grown seedlings gene expression affected by elicitor treatment A,B) *PIN2* gene expression affected by ethephon and MeJA. C,D) *PPOF* expression affected by treatment. E,F) *PRI* expression, G,H) *ERF1* expression. Each data point represents the mean±SE of three replicates. Means followed by different letters are significantly different at P= 0.05.

**Fig. 6.** Gene expression affected by wounding A) *PIN2* expression by wounding on control plants  B) *PIN2* expression by wounding on ethephon treated plants. C) *PIN2* expression by wounding on MeJA treated plants. D) *ERF1* expression not induced by wounding. E) *PRI* expression by wounding. Each data point represents the mean±SE of three replicates. Means followed by different letters are significantly different at P= 0.05.
Fig. 1.

A) Graph showing tomato plant height (cm) for different conditions: Control, EtOH, Ethephon, and MeJA. The graph compares Nr, RU, def1, and CM genotypes.

B) Photographs of tomato plants grown under different conditions: MS medium, MS +EtOH, MS +Ethephon, MS +MeJA, MS +MeJA +Ethephon. The plants show variations in growth and root development.

C) Graph showing hypocotyl length (cm) for different conditions and genotypes: Nr, RU, def1, CM. The graph includes Control, EtOH, Ethephon, MeJA, and MeJA +Ethephon.

D) Graph showing root length (cm) for Nr, RU, def1, CM genotypes under different conditions: Control, EtOH, Ethephon, MeJA, MeJA +Ethephon.
Fig. 2.
Fig. 3.
Fig. 4.
Reference


Agrawal AA (1999) Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80: 1713-1723


Campos ML, de Almeida M, Rossi ML, Martinelli AP, Litholdo CG, Figueira A, Rampelotti-
Ferreira FT, Vendramim JD, Benedito VA, Peres LEP (2009) Brassinosteroids interact negatively with jasmonates in the formation of anti-herbivory traits in tomato. Journal of Experimental Botany 60: 4346-4360

Chandok MR, Ekengren SK, Martin GB, Klessig DF (2004) Suppression of pathogen-inducible NO synthase (iNOS) activity in tomato increases susceptibility to Pseudomonas syringae. Proc Natl Acad Sci USA 101: 8239-8244


Navarro L (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin
signaling. Science 312: 436-439
Tian D, Tooker J, Peiffer M, Chung S, Felton G (2012b) Role of trichomes in defense against
herbivores: comparison of herbivore response to woolly and hairless trichome mutants in tomato (Solanum lycopersicum). Planta: 1-14
Tornero P, Gadea J, Conejero V, Vera P (1997) Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. Mol Plant Microbe Interact 10: 624-634
Ethylene negatively regulates local expression of plant defense lectin genes. Physiologia Plantarum 104: 365-372

Appendix: Herbivory in the previous generation primes tomato for induced defense

Introduction

As sessile organisms, plants have evolved diverse strategies to continuously adjust their responses to a broad range of abiotic and biotic stresses including herbivore challenges. The mechanisms of induced plant defense have been well studied (Ryan et al. 1986; Walling 2000; Wasternack et al. 2006). To avoid the herbivore attack, plants must immediately respond to the challenges with various defense strategies (Green and Ryan 1972). Among the strategies, the synthesis of the secondary metabolites in plants is a powerful chemical weapon because they often function as toxins to inhibit the herbivore's nutrition and growth (Lawrence et al. 2008; Maffei et al. 2007). Besides these chemicals weapons, plants also attract predators and parasitoids of the herbivore through the release of volatile organic compounds (VOCs) (Arimura et al. 2005). Trichomes are an example of physical defenses and may be inducible in some plants such as tomato (Kang et al. 2010). Transgenerational plant defense means the offspring’s defense traits are acquired by the adult parent; in other words the adaptation of the maternal effects responding to the environment can improve the fitness and immunity traits of the offspring. Several studies have examined the contribution of maternal stress to offspring’s phenotype and fitness (Agrawal 2001; Slaughter et al. 2011).

Tomato (Solanum lycopersicum), as a model to study plant defense, had been extensively studied (Arimura et al. 2005; Liechti et al. 2006; Wasternack et al. 2006). To test whether the progeny of tomato plants exposed to herbivory or MeJA treated are more resistant to herbivores than those of undamaged naïve plants. We used Micro-Tom tomato and Helicoverpa
zea (\textit{H. zea}) as a model to study whether insect fed or MeJA treatment could produce the progeny, which have more resistance.

**Materials and methods**

**Plants and insects**

Tomato seeds (cv. micro-tom, originally purchased from Tomato Growers Supply Co, Fort Myers, FL) were grown as described (Peiffer and Felton 2005), in Metromix 400 potting mix (Griffin Greenhouse & Nursery Supplies Tewksbury, MA) in greenhouse at Penn State University, University Park, PA, USA). The greenhouse was maintained on a 16-h photoperiod. For all induction experiments, plants were maintained in the greenhouse under 800W Super Spectrum lights (Sunlight Supply Co., Vancouver, WA). The \textit{H. zea} eggs were purchased from BioServ (Frenchtown, NJ) and reared on a wheat germ and casein-based artificial diet (Chippendale 1970) with ingredients from Bioserv in the Entomology Department, Penn State University (University Park PA, USA).

**Tomato leaf and fruit induced defense by MeJA**

To compare the long term induced defense MeJA on leaf and fruit. When plants have small fruits, we use 2.5mM MeJA in 0.8% ethanol spray on tomato plants. The control is no spray and spray with 0.8% ethanol. After 10 days spray took the leaves and fruit stages for bioassay. Neonates fed on artificial diet for one day and then transfer on the leaf to feed for 6 days in the cup. Compare the insect weight by precision balance.

At same time 50mg leaves and fruits were separately sampled for polyphenol oxidase (PPO) assay and 100mg tissue for gene expression assay. PPO assay was followed by Felton (1992) described. Briefly described as grind tissue in liquid nitrogen and immediately extract by 0.1M KPO$_4$ with 5% PVPP, sit on ice for 5minutes and centrifuge at 4 °C for 10minutes.
Immediately measure the absorbance at 450nm of the reaction with 5µl sample and 200µl 3M caffeic acid. The activity was expressed as OD/min/mg tissue.

**Transgenerational induced defense**

Previous studies showed insects feeding or mechanical damage induced the plant defense. To investigate the effect of parent plants treatment on next generational defense, we challenged the parent plants with *H. zea* fed, mechanical wound or MeJA treatment. Six week old plants (flower stage/fruit) were challenged by 4th instar *H. zea* randomly feeding for three days or mechanically wound small fruit. 2.5mM MeJA sprayed on plants as MeJA treatment. Harvest damaged or sprayed fruits and get seeds for F1 seeding trichome, bioassay and wounding response experiments. Neonates fed on artificial diet for one day and then transfer on the leaf to feed for 4 days in the cup. Compare the insect weight by precision balance.

When seedling had 4 fully expanded leaves, glandular trichomes were count on the upper side of the 4th leaf. Two leaf disks (0.6 cm diameter) were punched out beside the mid-vein. Numbers of type VI trichomes were counted using a dissecting scope. We mechanically wound the 4th leaf to compare the defensive response in the next generation plants. After 24 hours wound, the leaf were sampled for RNA extraction and quantitative RT-PCR.

**Results and Discussion**

The inducible defenses are regulated by plant hormones (Berger and Altmann 2000; Erb et al. 2009), such as jasmonic acid (JA), which is rapidly synthesized from a fatty acid precursor following herbivore attack. JA regulated defenses play a key role in the induction of plant defenses against chewing herbivores. Exogenous application of MeJA also induces a series of defense responses (Singh et al. 2008). In tomato, JA-response proteins include proteinase inhibitor 2 (*PIN2*) and polyphenol oxidase (*PPO*) (Angelini et al. 2008; Wasternack et al. 2006).
It has been shown that their induced gene expression is correlated with enhanced abundance of their protein products, which inhibit insect growth. In this experiment, two weeks after we sprayed plants with 2.5mM MeJA at the flowering/green fruit stage, the bioassay with leaves and fruits showed significantly reduced *H. zea* growth compared to control plants (*F*=31.41, *p*<0.01) (Fig. 1a). MeJA treatment significantly induced polyphenol oxidase (PPO) activity compared with the control plants on both leaves and fruits (Fig. 1b). Gene expression of *PIN2* showed that the MeJA could induce the expression on both leaves and fruits (Fig. 1c). It is confirmed that the induced protein activity and gene expression is correlated with enhanced abundance of their protein products, which inhibit insect growth.

Transgenerational plant defense means that the offspring’s immunity traits are acquired by the adult parent; in other words the adaptation of the maternal effects responding to the environment can improve the fitness and immunity traits of the offspring. Several studies have examined the contribution of maternal stress to offspring’s phenotype and fitness. Agrawal and colleagues used wild radish (*Raphanus raphanistrum* *L.* *brassicaceae*) and insect herbivory as model to examine induced defenses in next generation, and they found insect feeding on the maternal wild radish caused progeny to be more resistant than progeny from unfed wild radish (Agrawal 1999). Field experiments showed that plants exposed to herbivory in the early season had 60% higher relative fitness than un-exposed controls (Agrawal 1999). In some instances, the offspring of the damaged plants showed higher numbers of trichomes compared to the offspring from their undamaged parents (Agrawal 2001). A similar phenomenon was also observed in the progeny of yellow monkey flower, *Mimulus guttatus*, when the parents were exposed to herbivores (Holeski 2007). Transgenerational induction of high trichome density could confer a
selective advantage, if offspring are likely to experience the same herbivore pressure as their parents.

In this study, after plants were challenged by insects or MeJA treatment, the plants were allowed to continue growth and produce seeds for the next generation (F₁). These seeds were planted to test their response to insect feeding. At the 4th leaf stage, the 4th leaves were used to do bioassays with neonates to identify the inherited increase in herbivore resistance. All three treatment decreased growth of *H. zea* on the progeny of treated tomato plants by about 40% relative to controls (Fig. 2a, F=11.269, p<0.001). Preliminary experiments also showed that the small amount of ethanol used to dissolve MeJA for elicitation experiments has no subsequent herbivory.

At 4th leaf stage, the top fully expanded leaf glandular trichomes were counted using a dissecting microscope. The result showed the glandular trichomes number on the progeny of parent plants treated with MeJA or *H. zea* feeding, increased significantly compared to untreated plants (Fig. 2b F=6.61, p<0.001). These results were consistent with the previous study on wild radish and monkey flower. Both studies provide strong evidence that transgenerational defense can be induced by herbivory or MeJA treatment.

In another experiment to determine if MeJA could prime F₁ plants to respond more rapidly to wounding, at the 4th leaf stage, mechanical wounded the 4th leaf on F₁ plants whose parents were treated with MeJA. Twenty-four hours after wounding, the wounded leaf was assayed for the expression of proteinase inhibitor 2 (*PIN2*) using quantitative Reverse Transcription PCR (qRT-PCR). The wounded leaf from the MeJA treatment showed the highest level of *PIN2* expression and was significantly higher than all other treatments (Fig. 2c). These results indicate that the induced transgenerational defense is likely to result from a combination
of increased trichome density and more potent defensive responses to herbivore or wounding. These results are consistent with recent work (Luna et al. 2011; Slaughter et al. 2011) demonstrated a similar transgenerational resistance phenomena in response to priming-inducing by plant pathogens, salicylic acid (SA) or beta-aminobutyric acid (BABA). Both of these findings demonstrate that the induced defense can be transmitted to following generation. Transgenerational defense is heritable, transient and potentially regulated by epigenetic modifications, while the short-term induced defense is reversible when the stress is removed. Taken together, transgenerational stress immunity may provide a novel and useful tool for crop management and protection (Rasmann et al. 2012).
Figure 1. Induced defense by MeJA on leaf and fruits a) No-choice bioassay of *H. zea* growth affected by MeJA on leaf and fruits. b) PPO activity affect by MeJA treatment c) *PIN2* expression affected by MeJA treatment on leaf d) *PIN2* expression affected by MeJA treatment on fruit
Figure 2. Transgenerational resistance in tomato  a) Helicoverpa zea growth on tomatoes originating from parents that were either left undamaged (control) or subjected to caterpillar feeding, MeJA treatment, or mechanical damage. b) Trichome production on F1 tomato leaves. Trichomes were counted on the upper side of fully expanded 4th leaf. Plants were originating from parents that were left undamaged (open bars) or subject to H. zea feeding, MeJA application or mechanical damage (black bars).c) Effect of wounding on PIN2 gene expression F1 plants. The parent plants of F1 treated with MeJA. PIN2 measured 24 h after wounding damage.
Reference

Agrawal AA (1999) Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80: 1713-1723
Lawrence SD, Novak NG, Ju CJT, Cooke JEK (2008) Examining the molecular interaction between potato (Solanum tuberosum) and Colorado potato beetle Leptinotarsa decemlineata. Botany-Botanique 86: 1080-1091
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