LEPIDOPTERAN LARVAL SALIVARY SECRETIONS AND THEIR EFFECT ON TOMATO PLANT DEFENSES

A Thesis in
Entomology

by

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ABSTRACT

Forty-five percent of the over one million described insect species feed on plants. Agricultural losses due to arthropod pests account for about 100 billion dollars annually. Investigation of the role of caterpillar saliva is motivated by the long-term coevolutionary relationship between plants and the herbivores that consume them. Over the past 35 years it has become increasingly apparent that a wounded plant undergoes a variety of significant changes in plant chemistry and these changes affect the palatability of the plant as well as the fitness of the herbivore. Many plant defenses are activated and these include the accumulation of protease inhibitor genes. The cost related to these defenses provides the selection pressure behind the evolution of inducible defenses. Using genetic engineering in conjunction with these naturally occurring genes can lead to a more environmentally friendly pest management solution. This work is an important contribution towards investigating a cost-effective, environmentally safe solution to the insect herbivores’ role in the loss of crops in agriculture.

In this research I have investigated seven lepidopteran larvae: *H. zea, H. virescens, S. frugiperda, S. exigua, O. nubilalis, T. ni* and *M. sexta*. Saliva initiates digestive processes and may also be the first line of defense against microbes as well as plant defenses. Salivary glucose oxidase is primarily produced in the labial glands, and many entomologists currently believe it helps to suppress toxins produced by plants that are triggered by herbivore feeding. Herbivores acquire their amino acid nutrition from plant proteins. Many plant defense genes such as protease inhibitors block amino acid uptake in the feeding herbivore depriving it of the nutrition it needs. Protease inhibitor proteins are found in all forms of life and are one of the most plentiful classes of proteins found in the living world. In 1972, Green and Ryan were the first to discover the
accumulation of protease inhibitors after wounding. Tomato has been widely studied as a model system for investigating the mechanism of induced resistance based on its’ response to wounding and herbivory. Tomato leaf inhibitor II is a protein that acts as a potent inhibitor of endopeptidases displaying specificity towards trypsin and chymotrypsin proteases; it accumulates in the leaf as a result of insect attack. Jasmonates are a class of oxylipin molecules that are the best-characterized class of elicitors involved in the defensive responses of plants to wounding and herbivory. Ethylene, hydrogen peroxide (H$_2$O$_2$), oligogalacturonides (OGAs), fatty acid-amino acid conjugates (FACs), and abscisic acid (ABA) along with UV light activate jasmonic acid (JA) formation. Salicylate and nitric oxide (NO) repress JA formation. Local wounding along with prosystemin causes systemin to bind to the SR160 receptor. This activation of systemin along with the activation of JA leads to pin2 formation via the octadecanoid pathway. Protease inhibitor genes provide a new perspective on improving plant defense systems through two routes: either through using traditional breeding methods in order to select lines with enhanced expression or through the use of DNA technologies for the direct transfer of inhibitor genes to target specific digestive enzymes of pests and/or pathogens of plants.

The systemic nature of induced plant responses can be compared with the vertebrate immune response based on the manner in which endocrine signals target several tissues through the circulatory system. The polypeptide systemin has been isolated from tomato leaves and was found to be a powerful signal for systemic defense as well as inducing and regulating over 20 defense genes. Choice tests have been used to answer a broad array of research questions pertaining to behavior. Researchers try to obtain an understanding of insect feeding preference because understanding it is critical to the fields of entomology and ecology.
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Chapter 1

Caterpillars, their Saliva, Genes and how it’s all Related

This work is important towards investigating a cost-effective, environmentally safe solution to the insect herbivore’s role in loss of crops in agriculture. The human population growth has led to hunger problems in underdeveloped countries. As the average amount of cultivated land per capita has steadily declined, there has been an increase in hunger and lack of food worldwide. Substituting vegetal proteins in feed is more cost efficient than using animal proteins. Forty-five percent of the over one million described insect species feed on plants (Schoonhoven et al., 2005; Zheng and Dicke, 2008.) Agricultural crops experience losses of up to forty-five percent due to pests, diseases and competition by weeds. These losses account for about 100 billion dollars annually, and the majority of this damage is due to arthropods (Carlini and Grossi-de-Sa, 2002.)

The investigation of the role of caterpillar saliva in inducing plant defenses is motivated by the long-term coevolutionary relationship that exists between plants and the herbivores that consume them. Examining the feeding habits of caterpillars and taking into consideration that many of the plants’ defenses are triggered in the leaves led to my hypothesis that the caterpillars that are primarily leaf feeders should induce a stronger defensive response than caterpillars that feed on other areas of the plant. In this research, I explored this hypothesis by investigating the following objectives: the role of lepidopteran larval saliva as well as salivary gland homogenate in inducing plant defenses; if a systemic effect occurs; the role of glucose oxidase in each of the seven lepidopteran larval species; and the role of protease inhibitors in feeding preference.
Chemicals found in saliva that induce plants to turn-on wound-induced genes motivate investigating the systemic effect (Felton and Eichenseer, 1999; Felton, 2008.) This wound response can occur in the systemic leaves as well as at the injury site. Investigating the presence of salivary glucose oxidase is motivated by the previous work done with the corn earworm, where glucose oxidase was shown to be a major component of caterpillar saliva that interacts with plant defenses. Over the past 35 years it has become increasingly apparent that a wounded plant undergoes a variety of significant changes in plant chemistry and these changes affect the palatability of the plant as well as the fitness of the herbivore. This motivates the choice tests performed in the preference studies in order to gain an understanding of the role of induced defenses such as protease inhibitors in feeding preference. I have broadened these previous studies to compare seven caterpillar species that that feed on Solanaceous plants including tomato. The role of their salivary secretions in inducing defenses in tomato was investigated.

Insect-Plant Interactions

Plants may use signals to attract the pollinating insects they need, while other insects may respond to these signals and feed on the plant. An example of this can be seen in the monarch butterfly. The monarch butterfly is attracted to the milkweed plant and pollinates it; in turn the monarch butterfly lays eggs on this plant, which turn into voracious caterpillars that feed on the milkweed plant. Insects are versatile evolutionary innovators. Caterpillar feeding damages plant tissue, which results in triggering a plant’s defensive arsenal of responses, but insects in turn develop countermeasures to these defenses. Wounding alone isn’t solely responsible for the plant’s response to herbivore feeding. Elicitors may be present in the faeces or oral secretions of the feeding insects (Felton, 2008)
“Indeed the plant-herbivore ‘interface’ may be the major zone of interaction responsible for generating terrestrial organic diversity” (Ehrlich and Raven, 1964.) There are many examples of caterpillars overcoming barriers of evolution in order to exploit plants. *Trichopterus parvulus* caterpillars consume the sticky secretions used by sundew plants to catch insects. They then consume the plant and use it as a safe place to pupate; the sticky secretions from the plant protect the vulnerable pupae. *Synchloara* caterpillars use their silk to attach petals to their dorsal side to disguise themselves from parasitoids (Eisner, 2003.) Other insects have also been known to manipulate plants in other ways. For example, when aphids feed they trigger the plant to mobilize extra sugar and nutrients to the wound site, which is beneficial to the aphid (Spice, 2002.) Plants are also capable of synthesizing volatiles that attract natural enemies of the herbivorous insects (Alborne et al., 1997, 2000; Eisner, 2003; Zhu-Salzman et al., 2005.) “Plants are equipped with an arsenal of defenses ranging from chemical poisons to feeding deterrents to proteins that can block the activity of the insects digestive enzymes” (Irwin, 1997.) A vast array of studies have documented that both direct and indirect plant defenses are induced along with inducible physical defenses as a response to herbivory as well as a variety of other biotic stresses (Arimura et al., 2005; Frost et al., 2008; Leitner et al., 2005; van Dam et al., 2004.) Direct defenses involve the production of toxic chemicals, for example nicotine production, or affect nutrient uptake, as is the case for protease inhibitors (Wang et al., 2007.) Indirect defenses include the release of volatiles or nectar rewards in effort to attract natural predators of the feeding herbivore (Wang et al., 2007.) Plants have a variety of defenses to protect themselves against herbivores. While some of these defenses are pre-existing, others are induced by herbivore feeding and there is a cost related to these induced defenses (Kormneef and Pieterse, 2008.) The trade-off involved in induced defenses allows the plant to avoid the costs involved with direct defenses, but the plant will suffer damage in the time elapsed between herbivore attack and accumulation of defense (Frost et al., 2008.) Plants generally express their
full complement of defenses only in response to herbivore attack because of the cost associated with them (Baldwin, 1998; Wang et al., 2008; Zavala et al., 2004.) These induced defenses have been found in undamaged systemic leaves as well as the damaged localized leaf (Schilmiller and Howe, 2005; Wang et al., 2008; Wasternack et al., 2006; van Dam et al., 2001.)

When certain caterpillars such as the beet armyworm feed on a plant they release a substance called volicitin in their oral secretions that alert the plant of its’ feeding (Alborn et al., 1997). The plant in turn releases a signal to parasitic wasps that attack the caterpillar. Selection pressure would not allow caterpillars to keep volicitin as a component of their regurgitant if there was no fitness benefit to the caterpillar. It is suspected that volicitin may interrupt a process that allows plants to keep their leaves from being digestible (Irwin, 1997.) These are only a few of many examples of insect-plant coevolution.

Interactions between herbivores and the plants they feed on has often been referred to as warfare, and the interactions of plant and herbivore defenses has often been termed a coevolutionary arms race (Berenbaum and Zangerl, 2008; Gonzalez and Nebert, 1990; Musser et al., 2002a; Schoonhoven et al., 2005; Whittaker and Feeney, 1971.) To better understand coevolution, one must examine the patterns seen in these insect-plant interactions. During the Cretaceous period there was a burst of insect speciation (Zhu-Salzman et al., 2008.) The diversification of insects that can be seen today is due mainly to the stepwise pattern of coevolutionary changes superimposed on the pattern of variation seen in angiosperms (Ehrlich and Raven, 1964.) The type of coevolution that is the basis of insect-plant interactions has been described as “escape and radiate coevolution.” A plant species that is host to a diverse array of insects evolves defenses to rid itself of most of these herbivorous insects. This host plant succeeds in escaping most of these insects and the plant species goes on to diversify into several species sharing the same defense. The herbivorous insects then adapt to the plants’ defense. Giving rise to clades of specialized insect species associated with species in a particular clade of
plants (Futuyma, 1998.) An example of this coevolution can be seen in differences between specialist and generalist insects. Generalist insects are polyphagous, feeding on a variety of plant species. Specialist insects are oligophagous or monophagous; they are attracted to feeding on a particular type of plant and only feed on that. Insects that are specialists may contain higher amounts of detoxifying enzymes in the midgut than generalists. The cost related to these defenses provides the selection pressure behind the evolution of inducible defenses.

Using genetic engineering in conjunction with these naturally occurring genes can lead to a more environmentally friendly pest management solution. Plant genetic engineering technologies, when used in agriculture have resulted in insect-resistant crops (Gatehouse, 2008.) The use of transgenic cotton and maize resistant to larvae have reduced the use of pesticides in turn lowering production costs (Brooks and Barfoot, 2005; Gatehouse, 2008; Toenniessen et al., 2003.)

**Caterpillars Involved**

In my studies, I compared the role of salivary secretions in mediating plant defenses in seven caterpillar species. These species include *Spodoptera exigua*, which is a fruit and leaf-feeding generalist of family Noctuidae. The larva is commonly known as the beet armyworm. It is also sometimes referred to as the asparagus fern caterpillar. This species is native to Southern Asia and was introduced to Oregon in 1876 and again introduced to California in 1882, but now can be found worldwide (Berdegue, et al., 1998; Wilson, 1932.) *S. exigua* has been known to feed on over fifty plant species belonging to over ten families worldwide (Smits et al., 1987.) The adult moth of this species looks very similar to many other species but can be distinguished by the manner in which it holds its’ wings rolled around its’ body when at rest. In the United Kingdom this moth is well known as the small mottled willow moth.
Spodoptera frugiperda is another fruit- and leaf-feeding generalist of family Noctuidae. It is commonly known as the fall armyworm.

Trichoplusia ni is a leaf-feeding generalist of family Noctuidae. It is commonly known as the cabbage looper because it crawls inchworm-style arching its’ “back.” Loopers can be distinguished from inchworms based on their prolegs. Inchworms are of family Geometridae and have only two pairs of prolegs when loopers of family Noctuidae have three pairs of prolegs.

Manduca sexta is a leaf-feeding specialist of family Sphingidae. This larva is often called the tobacco hornworm because of the tail-like structure on its’ abdomen. These larvae feed on plants of the family Solanaceae. The adult of this species is often called the tobacco hawkmoth.

Helicoverpa zea is primarily a fruit-feeding generalist of family Noctuidae. It is most commonly known as the corn earworm but has also been called the American cotton bollworm and the tomato fruitworm. It is a polyphagous larva feeding on hundreds of host plants and the adult moth is a beneficial pollinator. Helicoverpa species are a common pest in Asia, Australia and the Americas. They have been known to develop resistance to chemicals such as DDT, organophosphates and pyrethroids (Volpicella et. al., 2003.) Helicoverpa zea is formerly of the genus Heliothis. Heliothis virescens is primarily a fruit-feeding generalist of family Noctuidae. It is commonly known as the tobacco budworm. The larvae of both species look very similar and it is often difficult to distinguish between the two; the orientation of the tubercle and microspines of the species is the best way to determine the difference between the two species.

The final species I examined was Ostrinia nubilalis, a stem and twig boring and fruit-feeding generalist of family Crambidae. It is commonly known as the European corn borer. This species was first found near Boston, Massachusetts in 1917; it has now spread west in the United
States and Canada to the Rocky Mountains and is found in many other countries

(http://edis.ifas.ufl.edu/IN313.)
<table>
<thead>
<tr>
<th>Species</th>
<th>Feeding Habits</th>
<th>Hosts</th>
<th>Damage</th>
<th>Distribution</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spodoptera frugiperda</td>
<td>Flower, fruit and seed feeder</td>
<td>Primarily grasses, sometimes vegetables and other</td>
<td>Tunnel into corn whorl and shred emerging leaves</td>
<td>Tropical Species, irregularly survives U.S. winters, can reach New England states</td>
<td>May bore into the head of leafy vegetables. Early-stage larvae feed as group and skeletonize leaves. Older larvae tunnel into plants.</td>
</tr>
<tr>
<td>Spodoptera exigua</td>
<td>Flower, fruit and seed feeder</td>
<td>Wide range, vegetables, crucifers, herbaceous, ornamentals, weeds</td>
<td>Primarily chew foliage, may chew stems and sometimes roots</td>
<td>Primarily Southern U.S., sometimes northern states</td>
<td>Common to North America, likes warm weather, adults migrate long distances</td>
</tr>
<tr>
<td>Trichoplusiani</td>
<td>Leaf Chewer</td>
<td>Wide range - especially vegetables, sometimes a greenhouse pest on ornamentals</td>
<td>Larvae chew leaves, later stages tunnel into plant</td>
<td></td>
<td>Young larvae feed on outer leaves, later stages feed more generally and tunnel</td>
</tr>
<tr>
<td>Manduca sexta</td>
<td>Leaf Chewer</td>
<td>Tomato and tobacco are favorites, some others</td>
<td>Chews leaves to defoliate plant quickly, sometimes eats green fruit</td>
<td></td>
<td>U.S. and southern Canada</td>
</tr>
<tr>
<td></td>
<td>Flower, fruit and seed feeder</td>
<td>Wide range, favors solanaceous plants, rarely a vegetable pest</td>
<td>Favors flower buds, tunneling of leaf bud may occur</td>
<td>Southern U.S., harsh winters kill pupae, some northern migration</td>
<td>Foliage rarely damaged, young larvae sometimes restrict to bud tunneling, later stages may consume entire bud</td>
</tr>
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<td>------------------------</td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td>Heliothis virescens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helicoverpa zea</td>
<td>Flower, fruit and seed feeder</td>
<td>Wide range, many vegetables, tomato seriously damaged</td>
<td>Larvae tunnel and bore into fruits and vegetables, also eat leaves, moths feed on nectar</td>
<td>Throughout North America, able to migrate and recolonize after harsh winters</td>
<td>One of the most destructive insects, On tomato, eggs are laid near flowers of developing fruit</td>
</tr>
<tr>
<td>Ostrinia nubilalis</td>
<td>Stem and twig borer</td>
<td>Wide range, ornamentals (no tomato?)</td>
<td>Larvae tunnel into stems, fruit and other plant parts</td>
<td>North America, East of Rockies</td>
<td>Can introduce plant pathogens and rot, early-stage larvae tunnel into leaf veins, later stages move into stalks or fruit</td>
</tr>
</tbody>
</table>
Saliva and the Glands that Produce it

“Saliva and the glands that produce it could be the leading edge of rapid evolutionary adaptation to new food sources” (Tabak and Kuska, 2004.) Saliva initiates digestive processes and may also be the first line of defense against microbes and plants. There are two pairs of salivary glands present in lepidopteran larvae: the mandibular glands and the labial glands. The labial glands are the main interest in this present research; they make silk in many species in addition to secreting saliva through a structure called the spinneret. Labial gland saliva contains many proteins. The long, tubular labial glands meet at a common duct and release salivary proteins at the spinneret, the same place that silk is secreted (Felton, 2008.) The mandibular glands do not secrete through the spinneret, but instead empty through pores in the mandible. Mandibular glands are also tubular but their size is variable among species of lepidopteran. These glands are often very small and only found in the head region, but in some species they may also extend well into the thorax of the caterpillar (Felton, 2008).

Salivary Glucose Oxidase

“Saliva is a remarkably complex fluid with an extraordinary natural history” (Tabak and Kuska, 2004.) The oral secretions of feeding herbivores play an eminent role in mediating plant defensive responses (Alborne et al., 2000; Korth and Dixon, 1997; Halitschke et al., 2001; Musser et al., 2002b; Peiffer and Felton, 2005.) Oral elicitors from herbivorous caterpillars fall
into two categories: fatty acid-amino acid conjugates (for example volicitin) and enzymes (for example \( \beta \)-glucosidase and glucose oxidase) (Mattiacci et al., 1995 and Peiffer and Felton, 2005.) Glucose oxidase, an enzyme commonly produced by fungi and other organisms, is also found in caterpillar saliva and helps to suppress toxins produced by plants that is triggered by herbivore feeding (Seedquest, 2002.) The glucose oxidase reaction is: \( \beta \)-D glucose + O\(_2\) → D-glucono-1,5 lactone + H\(_2\)O\(_2\). Glucose oxidase is a component of caterpillar saliva that assists the oxidation process of D-glucose producing D-gluconic acid and hydrogen peroxide (H\(_2\)O\(_2\)) (Bede et al., 2006; Felton and Eichenseer, 1999; Peiffer and Felton, 2005.) In caterpillars, glucose oxidase is primarily produced in the labial salivary glands, though less than 11% of the total enzyme activity has been detected in the mandibular glands and trace amounts have been found in the hemolymph as well as some other tissues (Bede et al., 2006; Eichenseer et al., 1999; Peiffer and Felton, 2005.)

Glucose oxidase has been shown to inhibit induced plant defenses (Bede et al., 2006; Eichenseer et al., 1999; Felton and Eichenseer, 1999; Musser et al., 2002a; Peiffer and Felton, 2005.) A study done at the University of Arkansas, investigated the role of glucose oxidase from the corn earworm as a specific component suppressing the plants’ defenses. Holes were cut in the leaves of tobacco plants to mimic caterpillar feeding. There were four different substances applied to these leaves: purified, active glucose oxidase; unpurified salivary gland extract; inactive glucose oxidase and water. The results showed that purified glucose oxidase and salivary gland extract suppressed the level of nicotine to the same level as an unwounded plant (Musser, et. al., 2002a). They also used a heated probe to cauterize the spinneret and prevent the caterpillar from salivating and therefore preventing the secretion of salivary enzymes. They found that caterpillars with intact spinnerets reduced nicotine levels when compared to caterpillars feeding without intact spinnerets (Musser, et. al., 2002a.)
Glucose oxidase converts sugar into gluconic acid and hydrogen peroxide (Seedquest, 2002.) This triggers a series of chemical reactions that cause a plant to change its chemical defenses. Plants produce toxins in response to caterpillar feeding causing the caterpillar to stop feeding. Glucose oxidase “tricks” the plant into reducing these “anti-insect” toxins which allows the caterpillar to continue feeding (Spice, 2002.)

The byproduct H$_2$O$_2$ is necessary for suppressing wound-inducible defenses of the plant (Peiffer and Felton, 2005.) H$_2$O$_2$ is generated in response to wounding. It is detected at wound sites as well as systemically within an hour and maxes at 4-6 hours, then declines. An oxidative burst is one of the earliest responses between pathogens and plants; H$_2$O$_2$ is part of the oxidative burst. Oxidative changes in the plant have been linked to oxidative damage in the insect midgut when the insect is feeding on a previously wounded plant (Orozco-Cardenas and Ryan, 1999.) Hydrogen peroxide is a downstream elicitor of jasmonic acid. JA synthesis and the production of H$_2$O$_2$ lead to the production of protease inhibitors. H$_2$O$_2$ acts as a secondary messenger for the induction of defense genes (Orozco-Cardenas et. al., 2001.) Hydrogen peroxide is an essential byproduct of the glucose oxidase reaction.

The antimicrobial properties of GOX can be attributed to the presence of H$_2$O$_2$. The antimicrobial properties of glucose oxidase and its enzymatic product, hydrogen peroxide were shown in an experiment involving the bacterial pathogens *Serratia marcescens* and *Psuedomonas auruginosa*. Caterpillars that had their labial glands extirpated and caterpillars that had a “mock” surgery were fed either no bacteria, diet that contained *S. marcescens* or diet that contained *P. auruginosa*. The caterpillars that were unable to salivate showed over fifty percent more mortality due to the bacteria-treated diet when compared to caterpillars that could salivate. This was the first time caterpillar saliva *in situ* proved to provide protection against bacterial pathogens (Musser, et. al., 2005.)
The hydrogen peroxide produced by glucose oxidase may interfere with signaling pathways by affecting signaling responses in the jasmonic acid or ethylene pathways. “The enzyme may cause the plant to react to more stresses other than insect chewing, stresses like temperature or pathogens” (Seedquest, 2002.) The gene that produces glucose oxidase in the corn earworm has been cloned and sequenced. When the gene is introduced into the plant genome, the plant became more resistant to pathogens and temperature stress. The developments found in glucose oxidase can lead to new methods such as a non-toxic chemical, genetically engineered plants resistant to the effects of the enzyme and a glucose oxidase treatment for plants infected with a virus or bacteria (Spice, 2002.) “And we certainly won’t be surprised if a fluid that people turn away from today comes to be appreciated for what it is: one of nature’s favorite genetics laboratories, and a source of lifesaving medical advances” (Tabak and Kuska, 2004.)

The regurgitant or oral secretions of herbivorous insects induce plants to turn-on wound-induced genes as well as trigger plants to release volatiles to attract parasitoids (Zhu-Salzman et. al., 2005.) This wound response can occur at the injury site as well as in the systemic leaves. Regurgitant is a product from the insect’s digestive system and is expelled through the mouth of the caterpillar. The oral secretions contain elicitors and to date five classes of elicitors of plant defenses have been chemically identified (Felton and Tumlinson, 2008.) These elicitors include fatty acid conjugates such as volicitin, peptides such as inceptin, disulfoxy acids such as caeliferins, bruchins and β-glucosidase. Four of these elicitors are associated with feeding and oral secretions while bruchins are associated with oviposition. Fatty acid conjugates, peptides and β-glucosidase have been isolated and identified from the oral secretions of lepidopteran larvae. β-glucosidase is the first reported herbivore elicitor; it is the only enzymatic elicitor of plant volatiles found in insects. The chemical structures show a diversity suggesting that plants in turn employ a diversity of mechanisms in order to detect and counteract against a broad array of herbivore defenses (Felton and Tumlinson, 2008.)
**Wound Inducible Protease Inhibitors**

Many plant defense genes limit the ability of the herbivore to acquire adequate amino acid nutrition from the plant. Using genetic engineering in conjunction with these naturally occurring genes can lead to an environmentally friendly pest management solution. When a plant is under attack from an herbivore, large-scale metabolic changes occur. These changes can be mimicked when wounds are mechanically produced and herbivore-specific elicitors are applied around the wound. Herbivory damages the tissue of the plant, but damaging the plant tissue is not always equivalent to herbivory due to the presence of herbivore-associated compounds. Based on the high metabolic and fitness costs associated with induced defenses the cascade of events leading to inducible defenses against herbivores should not be employed due to wounding caused by weather conditions such as wind, rain and hail (Mithofer, et. al., 2005.) A plant must be able to distinguish between mechanical damage disrupting the tissue and herbivore feeding for optimal fitness. Because plants respond to herbivory and artificial wounding differently, there may be a component of insect saliva that elicits signals indicating herbivore damage (Felton and Korth, 2000.) Producing defensive responses would only benefit the plant if it were being fed upon (for example volatile release.) But other responses such as the up-regulation of genes that are required for cell repair would benefit the plant in the case of herbivory or wounding due to weather events (Howe and Jander, 2008.)

In 1972, Green and Ryan were the first to discover the accumulation of protease inhibitors after wounding when studying both potato and tomato plants. Inhibitor I could be detected in the leaves of wounded tomato plants within the first twelve hours and continued to accumulate for at least 100 hours. The inhibitor I family of protease inhibitors are found in potatoes, tomatoes, beans and barley (Green and Ryan, 1972). The basis of the idea of protease inhibitors being protective agents against insects is that several of them are able to inhibit insect
digestive proteases (Green and Ryan, 1972.) Tomato has been widely studied as a model system based on its response to wounding and herbivory (Felton, 2005; Pautot et al., 1993; Sagi et al., 2004.) In tomato there are a plethora of proteins that are induced by insect herbivory. This array of proteins includes protease inhibitors, polyphenol oxidases, ascorbate oxidase, leucine aminopeptidase, arginase and threonine deaminase (Chen et al., 2004, 2005, 2007; Constabel et al., 1995; Felton et al., 1989, 1992.) These defensive proteins can be looked at, but they are JA-regulated and often follow similar profiles as pin2. Pin2 has been well characterized and documented to be affected by caterpillar feeding and is the reason I choose to focus on this particular gene in my studies. The octadecanoid or jasmonic acid pathway regulates the expression of many of these proteins (Howe et al., 1996; Li et al., 2002.)

The past 70 years of investigating plants has yielded the identification and characterization of five classical plant hormones: auxins, gibberellins, ethylene, absciscic acid and cytokinins that regulate the growth and developmental processes of plants (Ryan and Pearce, 1998). Based on the diverse ways in which arthropods may feed, arthropod inducible proteins (AIPs) are regulated by many molecules that play a role in wound signaling including systemin, oligosaccharides, jasmonates, salicylic acid, ethylene and abscisic acid (Leon et al., 2001, Zhu-Salzman et al., 2008.). In the tomato plant, wounding activates the release of a substance called a Protease Inhibitor Inducing Factor (PIIF). This wound signal initiates the accumulation of protease inhibitors (Ryan, 1978.) Protease inhibitor inducing factor activity is a component of the plant cell wall (Ryan, 1987.) Plant cell walls are mostly comprised of a mixture of polysaccharides and glycoproteins; the polysaccharides are made up of cellulose and complex carbohydrates (Ryan, 1988.) Oligosaccharides are produced in mass from the plant cell walls and are present at wound sites as early signals to induce gene products, such as protease inhibitors that enhance plant defenses. It has been suggested that there is an oligosaccharide-based system in plants that act as an early warning signal for defensive responses (Ryan, 1988.) These
oligosaccharides act as signals to activate the inducible arsenal of defensive responses in nearby cells (Ryan, 1994.). The oligogalacturonide (OGA) family of plant cell wall fragments along with the chitosan family of fragments are types of glycans shown to be active in the context of gene expression. OGA’s have been found to activate both phytoalexin and protease inhibitor synthesis as well as activating several other plant defensive responses (Ryan and Farmer, 1991.) OGA’s only act as elicitors of pin gene expression in tomato if the plants are wounded by insects or challenged by pathogens because they can secrete enzymes that are essential to the release of plant cell wall fragments. OGA’s can induce pin expression through ethylene-dependent and ethylene-independent pathways (Bowles, 1998.) Different size ranges of OGA’s induce rapid depolarization of membrane potential. It has been suggested that this is an early event in wound transduction.

Wounding caused by herbivory or by mechanical means releases protease-inhibitor inducing factors that travel throughout the plant to induce synthesis and accumulation of inhibitors I and II. These are powerful inhibitors of serine endopeptidases of the herbivores feeding on the plant and manipulate the plants’ nutritional quality to reduce it as a natural defense of the plants’ against attacking herbivores (Graham, et al., 1985a.) Tomato leaf inhibitor II is a protease inhibitor that accumulates in the leaf as a result of insect attack (Graham et al., 1985b.) Inhibitor II is a protein that acts as a potent inhibitor of endopeptidases; it displays specificity towards trypsin and chymotrypsin proteases (Graham et al., 1985b.) The synthesis of inhibitors in tomato leaf cells is cytoplasmic and accumulation of these mature proteins occurs in the central vacuole (Graham et al., 1985a.) The genes for these inhibitors introduce the possibility of identifying and isolating their wound-induced promoter regions. The promoter region is a region of DNA that acts like a switch to turn on or off the expression of genes. The gene expression may be manipulated in environmentally transformed plants via these promoter regions (Graham et al., 1985a.)
Herbivores and pathogens utilize proteases to digest plant tissues and obtain amino acids for their nutrition. Protease refers to both endopeptidases as well as exopeptidases; proteinase refers only to endopeptidases (Ryan, 1990.) In this review I will be using the term protease. Proteases are enzymes that degrade protein. Protease inhibitors form complexes with proteases and inhibit their proteolytic activity (Ryan, 1990.) When proteases are blocked by protease inhibitors the insect is unable to digest its’ food. These proteases may also have multiple functions in cellular metabolism, such as protecting fluids and tissues from foreign proteolytic activities degrading them. High concentrations of protease inhibitors are often found in particularly vulnerable fluids and tissues of the plant that are susceptible to foreign proteases. Insects damaging plant tissue increase the amount of protease inhibitors as a defense response of the plant. Attack by pathogens and viruses also induce protease inhibitor synthesis in plants. When a plant is wounded an increase in endogenous abscisic acid (ABA) and JA occurs leading to protease inhibitor II (pin2) gene expression (Farmer et al., 1992; Farmer and Ryan, 1992; Herde et al., 1999; Pena-Cortes et al., 1989, 1996.)

Protease inhibitor proteins are found in all forms of life and are one of the most plentiful classes of proteins found in the living world. The blood of higher animals cumulatively contains over 200 mg of different types of protease inhibitor proteins per 100 ml serum (Ryan, 1989.) There are two main natural functions of inhibitors: the prevention of uncontrolled proteolysis in cells, organelles and fluids; and to protect the proteins of these cells, fluids or tissues from introduced proteolytic enzymes (Ryan, 1989.) There are four classes of proteolytic enzymes: thiol/cysteine proteases, serine proteases, aspartic proteases and metalloproteases. The aspartic and thiol/cysteine classes are active in acidic conditions and serine and metalloproteases are active in alkaline conditions. Serine and cysteine proteases are primarily found in plants and are more relevant to their role in plant defenses. Only a few aspartic and metallo-proteases are found in plants (Ryan, 1990.) There is an abundance of different families of protease inhibitors.
including some that inhibit serine, cysteine and aspartate protease and metallocarboxypeptidases (Jongsma and Bolter, 1997.) The predominant digestive proteases of most caterpillars are serine proteases. The serine and cysteine endopeptidases (of beetles) are two classes of the digestive proteases identified in the guts of herbivorous insects that are the most likely targets for inhibition by engineered protease inhibitors. Though it has not yet been effective, these protease inhibitors have abundant potential towards improving plant defense through genetic engineering (Ryan, 1989.)

Protease inhibitors are the most often studied jasmonic acid –inducible proteins in tomato because they are expressed rapidly and systemically as a response to wounding and herbivore feeding. These protease inhibitors bind to and inhibit digestive proteases in the herbivores’ midgut. The negative effect of protease inhibitors on herbivores is a result of a compensatory response by the herbivore to overproduce digestive proteases, which depletes the essential amino acids resulting in decreased growth of the herbivore (Chen et. al., 2005.) A reduction in herbivore growth is not due solely to the action of protease inhibitors but involves several host compounds working in concert to exert a combination of toxic, antinutrititive and antifeedant effects. For example, wound-inducible polyphenol oxidases work together with protease inhibitors to reduce the nutritional quality of the plant tissue that is ingested (Chen et. al., 2005.)

There are other factors that play a role in the quality of an insect’s food. The majority of the research concerning the nutritional quality of an insect’s food emphasizes protein quantity neglecting the fact that protein quality is also critical to the growth and development of the insect (Bernays and Woodhead, 1984; Horie and Watanabe, 1983; Mattson, 1980; Schroeder, 1986; Srib, 1984.) Protein quality can be defined as the physical as well as the chemical properties that comprise the protein, for example, amino acid compliment and secondary and tertiary structures (Broadway and Duffey, 1988.) The structural configuration of the protein affects the digestibility of the protein (Broadway and Duffey, 1988.) Research supports the potential of
serine protease inhibitors in protecting plants against the insects that feed on them (Broadway, 1995; Broadway et al., 1986; Hilder et al., 1987; Johnson et al., 1989; McManus et al., 1994; Oppert et al., 1993; Orozco-Cardenas et al., 1993.) Protease inhibitors lower the proteolytic enzyme activity in a variety of insect species (Broadway, 1997; Christeller and Shaw, 1989; Wolfson and Murdock, 1987.) In tomato, serine protease mRNAs along with their corresponding proteins accumulate in the leaves of the plant as a response to wounding (Wingate et al., 1989.)

Plant protease inhibitors suppress the digestive enzymes leading to a reduction in the release of amino acids from food protein that can be utilized by insects (Zhu-Salzman et. al., 2005, and Volpicella et. al., 2003). Plant protease inhibitors inhibit the gut proteases in insects resulting in the stunt of their growth when incorporated into artificial diets or when expressed in transgenic plants the larvae feed on (Jongsma et. al., 1996.) This can delay the insect’s growth and development and eventually can lead to the insect’s starvation and death. The cost related to these defenses provides the selection pressure behind the evolution of inducible defenses.

*Pin2* is a wound-inducible serine protease inhibitor with two binding sites. “Inhibitor II genes code for ‘double-headed’ inhibitors that have amino acids at their P1 sites that are specific for both trypsin-like and chymotrypsin-like enzymes” (Ryan, 1989.) The P1 site is the reactive site where amino acids bind. The side chain of amino acids at the P1 site of the inhibitor determines its specificity. *Pin2* inhibits trypsin and chymotrypsin. Chymotrypsin is found in the midgut and is serine activated above a pH of 7 and can function in alkaline conditions.

The alkaline midgut of lepidoptera is where digestive enzymes are released and compounds which have been digested are absorbed. The lepidopteran midgut provides an optimal pH of between 8 and 11.5 for the activity of serine proteases as well as metalloexopeptidases (Jongsma and Bolter, 1997, and Ryan, 1990.) Jasmonic acid-regulated plant proteins affect the insect’s digestion by debilitating processes related to digestion and absorption. Microarray studies have shown an abundance of genes that are linked to these
proteins in plants and are up-regulated when feeding is detected (Zhu-Salzman et. al., 2008.)

Growth of leaf-feeding arthropods is dependent on their ability to obtain the ten essential amino acids from dietary protein (Jongsma and Bolter, 1997 and Chen et. al., 2007.) This requires several proteins to work together. Plants have evolved protease inhibitors that are resistant to proteolysis as well as being active under a diverse array of midgut pH levels (Jongsma and Bolter, 1997.) “Inhibiting dietary proteolysis through protease inhibitors may decrease access to essential amino acids” (Zhu-Salzman, et. al., 2008.) Digestive proteases catalyze the release of amino acids and peptides from ingested dietary protein. Protease inhibitors cause amino acid deficiency (Jongsma and Bolter, 1997.)

There are some issues concerning the effectiveness of protease inhibitors. They may take too long to affect the caterpillar. The caterpillar is still feeding and damaging the plant in the time it takes the protease inhibitors to take effect. There is a cost incurred to the plant to produce these defensive chemicals. Insects in turn develop countermeasures to these plant defensive responses. Digestive tracts often adapt to challenges of ingesting protease inhibitors and other anti-insect defense chemicals. Transgenic plants expressing protease inhibitors have been unsuccessful due to insect adaptation by overproducing digestive proteases or by selectively inducing inhibitor-insensitive proteases. Some *Helicoverpa* species have adapted to protease inhibitors by switching to proteases insensitive to the inhibitors. At the D20-Centre for plant breeding and reproduction research in the Netherlands, Jongsma et. al. performed a study in 1995 concerning the adaptation of *Spodoptera exigua* to plant protease activity by analyzing their gut protease activity. They found *S. exigua* adapts to exposure to protease inhibitors by the induction of new proteolytic activity that is insensitive to potato protease inhibitor II. The results they obtained indicate the presence of a control mechanism of response to protease inhibitors in diet that are dependent upon an array of proteases and can maintain a sufficient level of activity.
allowing normal growth and development. It is assumed that the mechanism directs the induction of gut proteases insensitive to plant protease inhibitors (Jongsma et. al., 1995.)

The protective function of protease inhibitors is due to a formation of stable complexes between the inhibitors and catalytic clefts of specified proteases that can block protein degradation. Enzymes mediate plant defenses that impair digestive processes in the insect midgut. While protease inhibitor enzymes are one part of the defense, arginase and threonine deaminase (TD) enzymes constitute the other part (Zhu-Salzman, et. al., 2008.) These jasmonic acid induced enzymes degrade the existing amino acids necessary to the insect, disrupting the insects’ digestion and most likely synergizing protease inhibitor activity. TD is an enzymatic defense molecule that is induced by wounding and is effective when present at lower concentrations. TD is essential for plant growth and development because of its role in producing isoleucine for protein synthesis. TD serves a duel role by participating in isoleucine biosynthesis in planta (tomato) and threonine degradation in the insect gut (Chen et. al., 2007.) Plant TD’s function in the chloroplast to catalyze steps in the biosynthesis of isoleucine. Isoleucine is required for synthesizing jasmonic acid-isoleucine, a signal essential for activating jasmonate-based defenses (Chen et. al., 2007.) There are many other enzymes involved in amino acid metabolism that are induced by herbivory that should be examined for their role in restricting amino acid absorption. A protein-stabilizing strategy has been proposed for the production of insect resistant plants. This strategy incorporates the idea of improving the anti-insect activity of toxic, proteolysis-susceptible proteins by administering a protease inhibitor that prevents the degradation of this toxic protein so it can utilize its defensive function (Zhu-Salzman et. al., 2008.)
**Signaling**

Different signals activate or repress JA formation. During tomato flower development the level of JA-isoleucine varies considerably implying JA-ile has a regulatory role (Hause et. al., 2000; Staswick and Tiryaki, 2004.) JA-ile is also essential for herbivore-induced defense signaling in tobacco (Paschold et. al., 2008.) JAR1 is an enzyme that activates JA by conjugating it to isoleucine. The identification of the mutant jar1-1 shows it is compromised in synthesizing JA-ile, demonstrating that this amino acid conjugate is essential in signaling (Staswick and Tiryaki, 2004.) JA-isoleucine is the mobile signal traveling through the vasculature of the plant activating *pin2* defense genes both locally as well as systemically.
Fig. 1-1: Model of Interactions Between SCF$^{\text{COI1}}$ and JAZ Repressor Genes. (A) JAZ repressors block transcription complexes (T). (B) In response to JA-ile SCF$^{\text{COI1}}$ interacts with JAZ repressors. (C) The SCF$^{\text{COI1}}$ interaction with JAZ repressors triggers JAZ ubiquitination (dark circles). (D) This ubiquitination causes JAZ to bind to the 26S proteasome leading to JAZ degradation and allowing the expression of early genes. Modified from Thines et. al., 2007.
COI1 (Coronatine Insensitive 1) is a coronatine-resistant mutant with a strong insensitivity to jasmonic acid. The phytotoxin coronatine is bacterially produced displaying jasmonate-like properties (Staswick and Tiryaki, 2004.) COI1 is an F-box protein that mediates jasmonic acid-isoleucine perception, playing a key role in herbivore resistance. F-box proteins are proteins containing at least one F-box motif, a protein structural motif of about fifty amino acids that mediate protein-protein interactions. F-box proteins are one of three components of the SCF complex, which mediates the ubiquitination of proteins targeted for degradation by the proteasome. The SCF complex (Skp, Cullen, F-box containing complex) is a multi-protein ligase complex catalyzing the ubiquitination of proteins destined for proteasomal degradation. Ubiquitination labels proteins for proteasomal degradation. COI1 plays an essential role in processes occurring downstream of jasmonic acid biosynthesis. COI1 stimulates JA-ile providing positive feedback on JA biosynthesis. The COI1 gene encodes an F-box protein subunit of the ubiquitin ligase SCF$^{\text{COI1}}$ that targets proteins in the JAZ family for degradation (Paschold et. al., 2008.) The JAZ (jasmonate ZIM domain) family of proteins are transcriptional repressors. ZIM (zinc-finger inflorescence meristem) is a putative transcription factor. COI1 regulates JA signaling by ubiquitin-mediated protein degradation (Devoto et. al., 2005; Pandey et. al., 2008.) Ubiquitination by the 26S proteasome is key to plant jasmonate responses. JA-ile promotes jasmonate-dependent degradation of JAZ proteins via the SCF$^{\text{COI1}}$ 26S proteasome pathway (Staswick, 2008; Thines et. al., 2007). Information is transferred from sensors to cellular responses in all eukaryotes by mitogen-activated protein kinases (MAPKs) (Koornneef and Pieterse, 2008.) Several of these MAPKs have been found to also play a role in plant signaling (Koornneef and Pieterse, 2008; Menke et al., 2004; Nakagami et al., 2005.) In addition to jasmonic acid, salicylic acid and ethylene are phytohormones recognized as regulating induced defenses and are key to the signaling pathways involved (Grant and Lamb, 2006; Harfouche et
al., 2007; Kahl et al., 2000; Lorenzo and Solano, 2005; Peng et al., 2004; Pozo et al., 2004; Van
Loon et al., 2006; Von Dahl and Baldwin, 2007; Ziegler et al., 2001.)
Fig. 1-2: Model of the Jasmonate Pathway. This diagram shows how wounding and elicitors affect the jasmonic acid pathway and initiate the accumulation of defense genes both locally as well as systemically. Modified from Wasternack et. al., 2006.
JA is an essential signaling compound in the responses of wounded plants, but is also regulated by many other signals. Ethylene, hydrogen peroxide (H$_2$O$_2$), oligogalacturonides (OGAs), fatty acid-amino acid conjugates (FACs), and abscisic acid (ABA) along with UV light activate jasmonic acid (JA) formation. Salicylate and nitric oxide (NO) repress JA formation (Orozco-Cardenas and Ryan, 2002.) Local wounding along with prosystemin causes systemin to bind to the SR160 receptor and activate jasmonic acid (Wasternack et. al., 2006.) This activation of systemin along with the activation of JA leads to pin2 formation via the octadecanoid pathway. The JA conjugate, JA-isoleucine, is generated locally and travels through the midvein of the leaf and through the plants’ vasculature system to the systemic leaf activating JA perception, in turn accumulating pin2 expression in the systemic leaf. Pin2 expression occurs exclusively in the mesophyll of the leaf when induced locally. Local JA biosynthesis is necessary for systemic JA perception. Systemic JA perception, but not biosynthesis is needed for defense gene expression (Wasternack et. al., 2006.) Hydrogen peroxide is also a powerful inducer of pin2 expression.

Oxylipins are biologically active components synthesized in response to herbivory, wounding and pathogen attack and are derived from the oxidative metabolism of polyunsaturated fatty acids (Howe and Schilmiller, 2002.) These oxylipins have a diverse array of functions in plants. Plants generate lipid-derived signals in response to stress; some of these signals include jasmonic acid, the methyl ester methyl jasmonate, the amino acid conjugates and the precursor 12-oxo-phytodienoic acid (OPDA) (Kessler and Baldwin, 2002; Miersch et al., 2007; Schilmiller and Howe, 2005; Halitschke and Baldwin, 2005.) As a response to plant tissue injury JA is synthesized from $\alpha$-linolenic acid (Chen et. al., 2005; Howe and Ryan, 1999; Halitschke and Baldwin, 2005; Miersch et al., 2007.) and is a potent activator of defense genes, inducing defensive protease inhibitors in plants (Ryan and Farmer, 1991; and Ryan and Pearce, 1998.) Jasmonic acid controls the expression of an abundance of target defensive genes as a response to tissue maceration. The pin2 gene that I have studied is heavily reliant upon the synthesis of
jasmonic acid. In the experiments I performed, mechanically wounding the plant with a cork borer, then immediately applying caterpillar saliva and/or salivary gland homogenate simulated insect feeding. Jasmonate responses are related to both plant stress and development. JA signaling dependent stress responses include not only defense against attacking herbivores, but also stress responses due to UV radiation, ozone and a variety of other abiotic stresses (Balbi and Devoto, 2008; Browse and Howe, 2008; Glazebrook, 2005; Wasternack, 2007.) In non-stressed plants jasmonates are also involved in a variety of processes such as carbon partitioning, mechanotransduction, senescence and reproductive development (Browse, 2005; Browse and Howe, 2008.)

Fatty acid-amino acid conjugates (FACs) have been found as a component of the oral secretions of many lepidopteran larvae of families Geometridae, Noctuidae and Sphingidae (Roda et al., 2004.) These FACs are both necessary and sufficient in inducing a JA burst as well as other JA-dependent responses (Halitschke and Baldwin, 2005.) Phytohormones such as jasmonic acid (and conjugates such as jasmonic acid-isoleucine) and ethylene mediate the elicitation of defenses during insect attack as well as the perception of insect-specific signals (Pandey et al., 2008.) In summary, the jasmonate pathway is regulated by many signals which can have a positive or negative effect; including systemin, oligogalacturonides, ethylene, abscisic acid, reactive oxygen species, electric signals, nitric oxide and insect-derived fatty-acid amino-acid conjugates (Schilmiller and Howe, 2005; Rojo et al., 1999.) Cross talk often occurs between the octadecanoid pathway and other pathways as a means to regulate these defenses (Lorenzo and Solano, 2005.) These other pathways may be regulated by: salicylic acid, nitric oxide, abscisic acid, ethylene, auxin and other secondary messengers for example hydrogen peroxide (Doares et al., 1995; Felton et al., 1999; O’Donnell et al., 1996; Orozco- Cardenas and Ryan, 1999; Pena-Cortes et al., 1991, 1995; Thipyapong and Steffens, 1997; Young et al., 1994.)
Systemic Wound Signaling

An injury to plant tissue activates defense mechanisms that occur not only locally but often in unwounded tissues as a systemic response (Rojo et al., 1999 and O’Donnell et al., 1998.) When a plant is injured a set of responses are activated including wound-responsive genes. The systemic response is generally defined as events occurring in distant unwounded leaves but may also occur in the stem, petioles and roots. Systemic responses may occur universally as over 100 plants species display systemic wound signaling as a result of herbivore attacks and pathogen response. This systemic wound signaling induces defensive chemicals in the leaves as well as stems (Ryan and Moura, 2002.). Local and systemic wound responses include metabolic changes as well as induction of gene expression (Leon et al., 2001.) In 1972, the first documented case of systemic resistance to herbivory, Green and Ryan performed experiments in which they allowed the Colorado potato beetle to feed on the leaves of tomato and potato plants. They discovered an accumulation of protease inhibitor I that occurred not only in the damaged leaves but also in the undamaged leaves, displaying a systemic effect. The systemic effect suggested that a protease-inducing factor was introduced into the plants’ vascular system when a leaf was wounded (Green and Ryan, 1972.) Protease inhibitor (pin) genes are often used as a marker because their expression is up-regulated systemically at the same rate as a local response to plant maceration and some data suggests it occurs even quicker (Bowles, 1998 and O’Donnell et al., 1998.)

When the plant cell wall is disrupted it releases fragments, which trigger a range of events related to developmental and defense responses. Glycans, lipids (e.g., JA) and peptide hormones play a role in the induction of pin gene expression in tomato (Bowles, 1998.) Systemin is an 18-amino acid oligopeptide found in tomato leaves that has shown to be an active elicitor of pin gene expression as a response to wounding and herbivory (Howe and Ryan, 1999.)
“Systemin is a wound hormone that plays a central role in regulating the expression of defense genes in plants” (Ryan, 2000.) Systemin is found in the phloem of the plant (Ryan and Pearce, 2003.) Prosystemin is a larger prohormone protein that is processed into systemin via proteolytic cleavages and serves as systemins’ precursor (Ryan, 2000.) Transgenic plants over-expressing prosystemin exhibit expression of wound-induced defense genes such as protease inhibitors and polyphenol oxidase (PPO) (Howe and Ryan, 1999.) “PPOs catalyze the oxidation of phenolic secondary metabolites into reactive quinones, which, in turn, polymerize into an insect-trapping glue or reduce the nutritional quality of plants by cross-linking proteins” (Baldwin et. al., 2001.)

The presence of systemin increases jasmonic acid in leaves of tomato plants. Systemin activates defense genes via the octadecanoid signaling pathway mediated by lipid-derived intermediates. Studies of the wound response pathway in tomato has shown that systemin along with its precursor prosystemin are components located upstream of the intracellular signaling cascade that require the activation and biosynthesis of jasmonic acid as a first line of defense signaling (Howe, 2004; Narvaez-Vasquez and Ryan, 2004.) The systemic nature of induced plant responses is similar to the vertebrate immune response in the manner in which endocrine signals target several tissues through the circulatory system (Howe, 2004.) In 1991 the polypeptide systemin was isolated from tomato leaves and was found to be a powerful signal for systemic defense as well as inducing and regulating over twenty defensive genes when supplied to the tomato plant (Ryan and Pearce, 1998, 2003; Ryan, 2000). Recently hydroxyproline-rich glycopeptides have been isolated from tomato leaves that have shown to be strong in their activation of protease inhibitor synthesis. A few of these peptides have been processed from a single 146 amino acid tomato polyprotein precursor (Narvaez-Vasquez, et. al., 2007.) These precursor genes show an expression similar to the systemin precursor prosystemin in tomato leaves in response to wounding and methyl jasmonate. The recent evidence implicates the
requirement for both the hydroxyproline-rich glycopeptides and the prosystemin gene to initiate a strong systemic response (Narvaez-Vasquez, et. al., 2007.)

Preference Studies

Choice tests have been used to answer a broad array of research questions pertaining to insect behavior ranging from studies concerning the environment and heredity to anthropogenic influences (Raffa et al., 2002.) The motivations behind these research questions can be basic investigations on the role of induced plant resistance on herbivores and parasitoids to management-oriented ecological motivations such as screening crop cultivars for resistant varieties (Raffa et al., 2002.) Researchers try to obtain an understanding of insect feeding preference because gaining an understanding is critical to the fields of entomology and ecology. Excised leaves or leaf disks are often used in these experiments because it is often impractical to use the entire plant (Risch, 1985.) Over the past 35 years it has become increasingly apparent that a wounded plant undergoes a variety of significant changes in plant chemistry and these changes affect the palatability of the plant as well as the fitness of the herbivore (Chen et al., 2007; Green and Ryan, 1972; Jongsma and Bolter, 1997; Kogan and Paxton, 1983; Rhoades, 1983; Risch, 1985; Schultz, 1983.)

Phenolics play a strong role in the survival of all plants as well as sometimes functioning as defensive molecules. “Phenylalanine-ammonia-lyase (PAL) is the first regulatory enzyme in the phenolpropanoid pathway that produces a variety of phenolics in plants” (Eichenseer et. al., 1998.) Chlorogenic acid is a phenolic acid common in plants and plays a role in influencing caterpillar host-plant selection. When chlorogenic acid is added to artificial diets it negatively
affects the growth and development of some lepidopteran species while completely unafflicting some other species. A study was done at the University of Arkansas by Eichenseer et. al. to investigate the role of chlorogenic acid in feeding (Eichenseer et. al., 1998.) When 3 mmol/disk (the amount that naturally occurs in leaves) of chlorogenic acid was added to glass fiber disks Manduca sexta showed no preference in feeding, but when 10 mmol/disk chlorogenic acid was added to the glass fiber disks it deterred M. sexta from feeding. This shows that M. sexta has adapted to accept the natural level of chlorogenic acid that occurs in plants but when the level of chlorogenic acid is elevated above the normal level it is less acceptable for consumption.

One of the few studies performed investigating feeding preference of undamaged vs. damaged leaves was performed at the Lincoln University in New Zealand by Hodge et. al. They investigated whether lepidopteran larvae preferred consuming leaves of the kawakawa plant that were undamaged when given the choice to consume either undamaged leaves or damaged leaves. The kawakawa plant does possess many anti-insect compounds (Hodge, et. al., 2000). In this study they utilized two different types of larvae and applied three different methods of damaging leaf tissue. They found there was no significant preference between the damaged and undamaged leaves.
Chapter 2

Methods

Plants and Insects

Tomato seeds (cv. Betterboy) were grown as described by Peiffer and Felton (2005) to the fifth node stage and the terminal leaflet of the fourth node was wounded and used in the experiments, as described below. Plants were grown and maintained in a greenhouse under 800W Super Spectrum Lights (Sunlight Supply Co., Vancouver, WA.) Lepidopteran larvae were obtained from various places. Eggs from larval species *S. exigua*, *T. ni* and *H. virescens* were obtained from the Tumlinson lab at Penn State University, Department of Entomology. Eggs from larval species *M. sexta*, *O. nubilalis* and *H. zea* were acquired from Benzon Research in Carlisle, PA. Eggs from larval species *S. frugiperda* were obtained from Corn Host Research Unit Insect-Rearing laboratory of the USDA-Agricultural Research Service in Starkville, MS. Each species of lepidopteran larvae were reared in a growth chamber at 27° C on a wheat-germ based artificial diet to fifth instars.

Saliva Preparation

To collect salivary secretions, fifth instar larvae were chilled on ice for at least fifteen minutes. Once completely immobile, the larvae were clipped into a metal hair clip and placed under a dissecting scope. As the caterpillar would warm to room temperature it would secrete saliva through the spinneret, which was collected using a gel-loading pipette tip (VWR, West
Chester, PA) containing glycerol. Saliva was collected from ten caterpillars with each tip and then stored in glycerol in an -80\(^\circ\) C freezer until needed. To prepare salivary gland homogenates, labial glands were dissected from caterpillars treated in a similar manner. The larvae were reared to fifth instars and placed on ice for at least fifteen minutes. The larvae were then placed in a dissecting tray under a dissecting scope while the labial glands were extracted. Ten pairs of labial glands per tube were stored in an -80\(^\circ\) C freezer until needed. To prepare the salivary gland homogenate, a tube of labial glands were first removed from the -80\(^\circ\) C freezer and stored on ice. Thirty ul PBS buffer per pair of glands was added to each tube and then homogenized using a motor pestle. PBS buffer is a phosphate buffer with saline; it has about the same physiological pH as the saliva and stabilizes the osmotic pressure. The PBS buffer concentration is 137 mM NaCl, 10 mM Phosphate and 2.7 mM KCl with a pH of 7.4.

**Treatment of Plants**

In order to test the effect of saliva on plant defenses, salivary gland homogenates or saliva collections were removed from the freezer, thawed and then applied to plants as described below. When applying saliva to plants, the collection from ten caterpillars was applied to each plant. The experiment had six treatments and six replicates per treatment equivalent to thirty-six plants. Twenty microliters of PBS was added to each tube containing saliva from 10 caterpillars so that the volume was sufficient to pipette on the leaf. Two holes were punched midvein in the terminal leaflet of the fourth leaf with a ¼ inch diameter cork borer. Immediately afterwards 20 \(\mu\)l of either PBS buffer (positive control) or saliva in PBS from *H. zea*, *H. virescens*, *S. frugiperda* or *T. ni* was applied around the edges of the wound sites. Control plants were left
completely untouched. After 24 hours the wounded leaves were harvested, frozen in liquid nitrogen and stored in an -80°C freezer for later mRNA extraction.

The aim of the labial gland experiments was to investigate the effect of saliva from seven lepidopteran larval species that are known pests of the tomato plant. Because saliva could not be collected from some of the insect species such as *M. sexta*, salivary gland homogenates were prepared to treat plants as a comparison to the saliva experiment. The Bradford assay was performed in order to determine the protein content of the labial glands of the various species of lepidopteran. The homogenized glands were diluted and adjusted with PBS to obtain 20 μg of protein in 20 μl of homogenate. Again the treatment leaves were left untouched as in the saliva experiment and the plants were also wounded in the same manner. PBS buffer (20 μl), the positive control or gland homogenate from *H. zea, H. virescens, S. frugiperda, S. exigua, T. ni, M. sexta* or *O. nubilalis* was immediately applied around the edges of the wound site of the leaflet. After 24 hours, the wounded leaves along with the systemic leaflets were harvested, frozen in liquid nitrogen and stored in an -80°C freezer for later RNA extraction. This experiment had nine treatments and nine replicates per treatment for a total of 81 plants and was replicated for a total of three experiments.

Leaf tissue (100 mg) was harvested from around the wound site of each leaf and was homogenized in liquid nitrogen. Messenger RNA was purified using an RNeasy Plus Mini-kit (Qiagen, Valencia, CA). Purified RNA (1μg) was used with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) to create cDNA. All reactions used Realtime PCR primers with 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. For statistical analysis, relative expression values
were analyzed by ANOVA (Minitab Inc., State College, PA) using Fisher’s and Tukey’s separation of means (P< 0.05.)

Fig. 2-1: Picture Showing Treatment Leaf and Cork Borer used in Wounding. The pink arrows indicate systemic leaves.
The systemic experiment used the frozen leaflets from the first gland homogenate experiment. The systemic leaflets are the two leaflets adjacent to the terminal leaflet. This experiment showed the best induction and would be expected to display the strongest systemic result if any occurred. The systemic leaflets were removed from the -80°C freezer and stored in liquid nitrogen. They were processed as described above.

**Detecting Glucose Oxidase**

Glucose oxidase plays an important role in plant-insect interactions as well as being well-known for its’ antimicrobial properties. Glucose oxidase has been found previously in the labial glands of *H. zea*. The aim of this experiment was to determine if it was present in six other species of lepidopteran larvae: *H. virescens, S. frugiperda, S. exigua, T. ni, M. sexta and O. nubilalis*. Labial glands were extracted and stored in an -80°C freezer as previously described. Labial glands from each species of larvae were removed from the -80°C freezer and stored on ice. Thirty μl of phosphate buffer (0.1M composition, pH = 7) was added to each tube per pair of glands and homogenized using a motor pestle. Three wells of a UV transparent plate were used for each species of lepidopteran. Five microliters homogenate was pipetted into each well. Two hundred microliters dianisidine mix was immediately added to each well and the plate was quickly loaded into the spectrophotometer and read. The glucose oxidase assay was performed at the same time as a Bradford assay to determine protein content. The dianisidine mixture was made by combining 2.6 mg dianisidine with 5 mls phosphate buffer (0.1M, pH = 7.), 1 ml D-glucose (100 mg/ml) and 40 μl horseradish peroxidase (1 mg/ml, Sigma #p2088.) A spectrophotometric glucose oxidase assay was performed to determine if glucose oxidase was present by measuring the change in absorbance at 460 nm to determine the specific activity, which is the enzyme activity per amount protein. (Eichenseer et al., 1999). The glucose oxidase
reaction: $\beta$-D glucose + $O_2 \rightarrow$ D-glucono-1,5 lactone + $H_2O_2$ is coupled with another reaction. It is coupled with horseradish peroxidase, which oxidizes dianisidine creating DABoxide and water causing a color change. The coupling of these two reactions uses oxygen and reduces it to hydrogen peroxide then reducing the hydrogen peroxide to water. We measure the DABoxide that is created using an indirect enzymatic reaction.

Preference Studies

These experiments were performed in order to determine the role of insect-induced responses in plants on larval feeding preference. *M. sexta* (a leaf-feeding species) larvae were given the choice of eating leaf disks from either completely untouched control plants, leaf disks from plants treated with *S. exigua* homogenized labial glands, a fruit-feeding larva, and leaf disks from plants treated with *T. ni* homogenized labial glands, another leaf-feeding larva. *S. exigua* treated plants have shown the highest level of relative expression of the *pin2* gene, a protease inhibitor, in previous experiments. The more protease inhibitors and defense genes that are induced and accumulate in a plant the less palatable it should be to a feeding caterpillar. Therefore the *M. sexta* larvae should be most attracted to consuming the control plant leaf disks and least attracted to consuming the *S. exigua* treated plant leaf disks. Salivary gland homogenates were applied to plants in the same manner as previously described. All plants used were in the six-node stage and the fifth terminal leaflet served as the treatment leaf. In the first experiment 45 plants were used, 15 were control and left untouched, 15 were wounded and treated with *T. ni* gland homogenate, and the remaining 15 were wounded and treated with *S. exigua* gland homogenate. In the second experiment, 15 plants were control and left completely untouched, 16 plants were wounded using a cut 200 $\mu$l pipette tip (diameter = 0.5 cm) and treated with *T. ni* gland homogenate as described previously. Sixteen plants were also wounded with a
cut 200 μl pipette tip and treated with S. exigua gland homogenate. In the third experiment there were 16 plants per treatment and they were treated in the same manner as the second experiment.

After 24 hours, leaf disks were taken from each treatment leaflet, as many disks as the remaining surface area would allow were taken from each leaflet. A #8 cork borer with a diameter of 1.2 cm was used to take leaf disks. Falcon six well plates from Bectin Dickinson Labware Company, NJ were used. These arenas measured 12.5 cm by 8 cm and were 1.8 cm high. Each arena plate contained six trial areas. Each trial area had a piece of packing foam fit into the bottom and a piece of filter paper was placed on top of the packing foam and moistened with water. In each trial area a leaf disk from each treatment was arranged randomly ensuring the order of the leaf disks was randomly different in each trial area. There were three leaf disks in
each trial area that were held in place by dissecting pins cut in half. A single *M. sexta* third instar larvae was placed in each trial area and allowed to feed for six hours. The arenas with the larvae were placed in a growth chamber at 27° C. Observations were taken every hour for the six-hour feeding period. After six hours the remaining disks areas were measured by scanning the images and measuring them using SigmaScan software (Jandel, San Rafael, CA, US.A.) The areas were calibrated against a ruler and uneaten leaf disks. The average percentage of leaf disk area consumed was calculated and statistical analysis was performed using Minitab (State College, PA) ANOVA, general linear model.

Three separate preference experiments were performed. In the first experiment, second instar *M. sexta* with slipped headcapsules were removed and starved for 24 hours. There were a total of 29 replicate areas per treatment in the first experiment. In the second experiment, second instar *M. sexta* larvae with slipped headcapsules were removed the day before and given new food. They were starved for six hours prior to the experiment. The second experiment had a total of 24 replicate areas. In the third experiment the *M. sexta* larvae were treated in the same manner as the second experiment and this experiment yielded a total of 27 replicates per treatment.
Chapter 3

Results

Saliva Experiment

Saliva was successfully collected from only Helicoverpa zea, Heliothis virescens, Spodoptera frugiperda and Trichoplusia ni; this may be attributed to the Noctuidae head structure, which allowed better access to the spinneret. This experiment was performed in September of 2006. There were six treatments and six replicates per treatment for a total of 36 plants.

Quantitative Real-Time PCR was used to determine the effects of the saliva treatments on defense gene expression by observing pin2 transcript levels. Quantitative Real-Time PCR compares the relative expression (RQ) values obtained pertaining to the induction observed of the pin2 gene being investigated. The relative expression level is indicative of the amount of transcript accumulation at a particular time. It is equivalent to the ratio of pin2 / ubiquitin because the expression level is expressed in terms relative to the non-induced levels. Ubiquitin was used as the reference gene to compare to pin2. The average RQ of the control plants is 0.818; this is acceptable because it should be approximately one. The control plants are uninduced and should not be displaying high levels of the gene of interest when compared to the housekeeping gene. The average RQ for pin2 in the PBS treated plants is 10.99; this shows that wounding induced the plants. The average RQ for pin2 in plants treated with H. zea or S. frugiperda saliva was slightly more than 1.0, which may suggest suppression of induced pin2 although this value was not significant (P> 0.05). The average RQ of the T. ni saliva treated plants was 20.6 which indicates that saliva from this species induced a response from the plant.
The average RQ obtained from the *H. virescens* treated plants is 18.5. This also shows the saliva induced a response from the plant because the average RQ value is higher than the PBS treated plants. This experiment showed generally low levels of induction, which may be influenced by lighting or UV effects. The saliva experiment yielded a P-value of 0.175, which is not statistically significant.

![Saliva Experiment](image)

**Fig.3-1: Saliva Experiment**
To determine the effect of larval saliva on defense gene expression in tomato, plants were mechanically wounded and treated with saliva from four species of lepidopteran larvae. Saliva was successfully collected from *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera frugiperda* and *Trichoplusia ni*. The PBS treated plants were mechanically wounded and PBS buffer was applied in the same manner as saliva to observe the effect of wounding and control plants were left untouched. 100 mg samples of leaf tissue were collected after 24 hours and *pin2* transcript levels were analyzed by quantitative real-time PCR using ubiquitin as a reference gene. The results presented are the average of six replicates. Values represent the mean. Bars indicate standard error. P-value = 0.175.
Labial Gland Experiments

The labial gland extract experiments were performed in addition to the saliva experiment because labial glands could be extracted from all seven species of interest where saliva could only be collected from four species. In addition there may be other proteins present in the labial glands that play a role in herbivore-mediated defenses. These extracted glands were homogenized in a PBS buffer with a motor pestle for use in the experiments. The first experiment was performed in October of 2006. There were nine treatments and three replicates per treatment for a total of 27 plants. The results showed good induction of pin2 but also some variability. The experiment was repeated twice with 9 replicates per treatment per experiment. The second experiment was performed in June of 2007 but the results indicated that plants were not strongly induced by any treatment including the positive control with PBS. There were nine treatments and nine replicates per treatment for a total of 81 plants. The third experiment was performed in July of 2007 with results indicative of normal wound-induced effects. There were also nine treatments and nine replicates per treatment for a total of 81 plants. The gland experiments were performed at three different times of year for a total of 21 replicates where induction may have been influenced by lighting or potential UV effects that are known to affect pin2 expression. Because the second experiment did not show significant induction, those results were not included in the graph below, Figure 3-2. Therefore the results for the first and third experiments, a total of twelve replicates were included in the analyses.

Quantitative Real-Time PCR compares the relative expression (RQ) values obtained pertaining to the induction observed of the pin2 gene being investigated. Ubiquitin was used as a reference gene to compare to pin2 transcript levels. The control plants yielded an average RQ of
0.888; this is acceptable because a value near one is expected. The PBS plants yielded an average RQ value of 24.78; this shows wounding induced the plants. The average for *H. zea* treated plants was 62.1, *H. virescens* treated plants RQ was 181.7, *T. ni* treated plants was 54.9, S. *frugiperda* treated plants was 97.8, *O. nubilalis* treated plants was 69.3, *S. exigua* treated plants was 251.4, and the *M.sexta* treated plants yielded 72.4. All the lepidopteran labial gland treatments showed an induced response because the average RQ is higher than the average RQ of the PBS treated plants indicating that the induction was not due solely to wounding alone. At a p-value of .05 the *S. exigua* and *H. virescens* treatments are statistically significantly higher than the PBS wounded control treatment. The results from the first and third experiments yielded a P-value of 0.004, which is statistically significant.
Labial Gland Experiments (1&3)

Tomato plant leaves were mechanically wounded and treated with labial gland homogenate from seven lepidopteran larvae. 100 mg samples of leaf tissue were collected after 24 hours and *pin2* transcript levels were analyzed by quantitative real-time PCR using ubiquitin as a reference gene. The results presented are the average of twelve replicates. The second experiment did not show significant induction and the results are not included here. Values represent the mean. Bars indicate standard error. P-value = 0.004.

**Fig. 3-2: Labial Gland Experiments (1&3)**

Tomato plant leaves were mechanically wounded and treated with labial gland homogenate from seven lepidopteran larvae. 100 mg samples of leaf tissue were collected after 24 hours and *pin2* transcript levels were analyzed by quantitative real-time PCR using ubiquitin as a reference gene. The results presented are the average of twelve replicates. The second experiment did not show significant induction and the results are not included here. Values represent the mean. Bars indicate standard error. P-value = 0.004.

**Systemic Induction Experiment**

To determine if salivary secretions could affect gene expression systemically, distal leaves were tested for the ability to express elevated *pin2* transcript levels. 100 mg of leaf tissue was analyzed from the two leaflets adjacent to the treatment leaf. The leaflets analyzed were from the first gland experiment because strong induction was observed in that experiment.
Quantitative Real-Time PCR was used to determine if elevated *pin2* transcript levels were present using ubiquitin as a reference gene. The values obtained were very low and variable. Figure 3-3 indicates that the degree of wounding and the application of saliva had no systemic effects on *pin2* expression. The lack of systemic induction observed here may relate to the relatively small amount of mechanical wounding involved with the removal of tissue as well as the tomato cultivar used. Unpublished data in our laboratory indicates that cv. Better Boy used in our studies shows less systemic induction than some other cultivars such as cv. Castlemart that have been frequently used in studies on systemic induction.

Investigating whether a systemic response would be induced by any of the species of lepidopteran of interest was worth looking into. The high levels of variation seen can be attributed to the extremely low levels of expression observed. A P-value of 0.250 was obtained, which is not statistically significant.
Salivary Glucose Oxidase in Caterpillars

Salivary glucose oxidase has been found in *Helicoverpa zea*. A spectrophotometric glucose oxidase assay was performed in order to determine if it is present in six other species of lepidopteran larvae. My results indicated that highest activity of GOX was observed in *H. virescens*, this species yielded a specific activity of 247.7 μmoles/min/mg protein. There was also a substantial amount of glucose oxidase activity in *H. zea*, a specific activity level of 43.3...
µmoles/min/mg protein was determined; and trace amounts were found in *S. frugiperda*, yielding a specific activity of 4.1 µmoles/min/mg protein. All of these larvae are fruit-feeding lepidopterans of family Noctuidae. Therefore the presence of glucose oxidase could be related to their fruit-feeding eating habits. These are also the species I was able to collect saliva from; they may have larger spinnerets better equipped to secrete saliva and therefore able to secrete more glucose oxidase leading to them producing more in their labial glands. Glucose oxidase was not detectable in the other species including *O. nubilalis, S. exigua, T. ni,* and *M. sexta.*

**GOX Assay**

![GOX Assay Diagram](image)

**Fig. 3-4:** Glucose Oxidase Activity in Salivary Secretions

A spectrophometric glucose oxidase assay was performed to determine if glucose oxidase was present by measuring the change in absorbance at 460 nm to determine the specific activity, which is the enzyme activity per amount protein. Values represent specific activity.
Preference Studies

These experiments were conducted in order to determine the role of defense gene expression in plants on larval feeding preference. *Manduca sexta* (a leaf-feeding larva) were placed in arenas with the choice of feeding on three leaf disks. One leaf disk was taken from an untouched control plant leaf. Another disk was taken from leaves from plants treated with homogenized glands from *Spodoptera exigua* (a fruit-feeding larva). The third leaf disk was taken from leaves of plants treated with homogenized glands from *Trichoplusia ni* (a leaf-feeding larva). In previous experiments, plants treated with *S. exigua* gland homogenate have shown the highest level of relative expression of the *pin2* gene, a plant defense gene. The more defense genes that are induced and accumulate in a plant, the less palatable it should be to a feeding caterpillar. Therefore, *M. sexta* larvae should have been most attracted to consuming control plant leaf disks and least attracted to consuming leaf disks from plants treated with *S. exigua* homogenized glands. *M. sexta* were allowed to feed for six hours. The remaining leaf disks were scanned into a sigma scan program and analyzed for average amount leaf loss.

In the first preference study, *S. exigua* treated leaf disks were the most preferred by *M. sexta* and the control leaf disks were the least preferred. The sigma scan results supported my hourly observations. In the previous labial gland experiments, the *S. exigua* treatment has shown to induce the highest level of *pin2* expression. This was expected to be the least preferred by *M. sexta* to consume; the control leaf disks should be their first choice because the leaf disks are uninduced. It is possible that because of the elevated level of *pin2* in the *S. exigua* leaf disks that affects on the digestive system cause larvae to consume more. Thus larvae consume greater amounts of foliage to compensate for the lower nutritional quality of induced foliage. The first preference study obtained a P-value of 0.084 indicating the results are marginally significant.
In the second preference study *T. ni* treated leaf disks were most preferred by *M. sexta* larvae and *S. exigua* treated leaf disks were least preferred. The sigma scan results support my hourly observations. The results from this experiment were different from the first experiment. Although *S. exigua* leaf disks are predicted to be the least preferred, and control leaf disks should be most preferred, *T. ni* leaf disks were most preferred. The P-value obtained is 0.195 indicating the results were not statistically significant.

In the third preference study the *T. ni* treated leaf disks were the most preferred. The *S. exigua* treated leaf disks and the control leaf disks were preferred almost the same. The control leaf disks were slightly less preferred. The sigma scan results support my hourly observations. This experiment yielded slightly different results from the second experiment. *T. ni* leaf disks were the most preferred in both the second and third experiments. It is possible that other defenses are involved. Other defenses may be induced blocking the amino acid uptake in the caterpillar resulting in the larvae consuming more. This experiment yielded a P-value of 0.001, which is statistically significant.

All three studies were combined and the results are shown in figure 3-5. Control leaf disks had an average percent leaf disk area consumed of 7.8819 percent. *S. exigua* treated leaf disks had an average percent leaf disk area consumed of 9.2571 percent. *T. ni* treated leaf disks had an average percent leaf disk area consumed of 13.9516 percent. *T. ni* treated leaf disks were the most preferred and the *S. exigua* treated leaf disks were the least preferred. The control leaf disks were expected to be preferred over the *T. ni* treated leaf disks because the control leaf disks were uninduced. When all three experiments were combined a P-value of 0.007 was obtained indicating that the induced disks were most preferred by *M. sexta* larvae.
Fig. 3-5: Preference Studies
These experiments were conducted in order to determine the role of insect-induced responses in plants on larval feeding preference. *M. sexta* (a leaf-feeding species) larvae were given the choice of eating leaf disks from either completely untouched control plants, leaf disks from plants treated with *S. exigua* homogenized labial glands, a fruit-feeding larva, and leaf disks from plants treated with *T. ni* homogenized labial glands, another leaf-feeding larva. The results presented are the average of eighty replicates. Bars indicate standard error. Values represent mean. P-value = 0.007.
Chapter 4

Discussion

The oral secretions of feeding herbivores play an eminent role in mediating plant defensive responses (Alborne et. al., 2000; Korth and Dixon, 1997; Halitschke et. al., 2001; Musser et. al., 2006; Peiffer and Felton, 2005.) Saliva initiates digestive processes and may be the first line of defense against microbes and plant defenses. Chemicals found in oral secretions may induce plants to turn-on wound-induced genes triggering plants to release volatiles to attract parasitoids as well as other natural enemies (Zhu-Salzman et. al., 2005.) Elicitors increase the expression of genes regulating the production of volatiles. Plants respond to wounding via different signals leading to defense gene expression. Among the chemical defense strategies plants utilize to protect themselves is the formation of protease inhibitors that affect the nutrient consumption of herbivores (Green and Ryan, 1972.) An injury to plant tissue activates defense mechanisms that occur not only locally but often in unwounded tissues as a systemic response (Wasternack et. al., 2006.) Local and systemic wound responses include metabolic changes as well as induction of gene expression.

Glucose oxidase (GOX) is primarily produced in the labial glands and has shown to reduce plant defenses. GOX converts sugar into gluconic acid and hydrogen peroxide. Hydrogen peroxide is an essential byproduct of the glucose oxidase reaction. GOX has been shown to inhibit plant defenses. It is well known for its antimicrobial properties and plays an eminent role in saliva reducing the infectivity of bacterial pathogens.
The arsenal of defenses plants are equipped with seems to override the defenses contained in the caterpillar saliva. Protease inhibitors are the most often studied jasmonic acid-inducible proteins in tomato because they are expressed rapidly and systemically as a response to wounding and herbivore feeding. Based on the labial gland experiment resulting in higher relative expression values in the homogenate treatments than the control and PBS (positive control) treatments, there was a response in the form of protease inhibitors (pin2) induced in the plant. Pin2 accumulates in the leaf as a result of insect attack affecting the palatability of the plant and the fitness of the herbivore. Herbivores acquire their amino acid nutrition from the digestion of plant proteins. The basis of the idea of protease inhibitors being protective agents against insects is that several of them are able to bind to and inhibit insect digestive proteases in the herbivores midgut. One could conclude that plants have evolved the better defensive mechanism than the caterpillars because they are able to recognize caterpillar feeding from the salivary secretions and respond to this by activating protease inhibitors. Protease inhibitors are consumed by the caterpillar and block the amino acid uptake of the caterpillar reducing the protein quality causing the caterpillar to become sick and eventually die of starvation. The cost related to the direct defenses of the plant to have these protease inhibitors proves to be worth incurring since the caterpillar will become ill and cease feeding.

Many plant defense mechanisms are located in the leaves of the plant and are often triggered by the many trichomes located in the leaf. The leaf of the plant is very important because the leaf area affects water absorption and photosynthetic processes that are key to plant survival and growth. This led me to hypothesize that leaf-feeding caterpillars would induce a stronger response because I thought the plants would be quick to recognize the saliva of these larvae. From the data obtained in the labial gland experiment I would have to reject my original hypothesis that leaf-feeding caterpillars induce a stronger response. The labial gland homogenate experiment resulted in higher relative expression values in the homogenate treatments than the
control and PBS treatments showing labial gland homogenate induced defense gene expression in the plant. The saliva of leaf-feeding caterpillars may have adapted to tomato plant leaf defenses better than caterpillars that typically feed on other areas of the plant therefore the saliva of leaf-feeding caterpillars is not as easily recognized by the plant. That may be why lower levels of induction were observed in leaf-feeding species. Fruit-feeding caterpillars had high levels of induction and this may be attributed to them not adapting as well to the plant defenses located in the plants’ leaf. One could conclude that plants have evolved the better defensive mechanism than the caterpillars because they are able to recognize caterpillar feeding from the salivary secretions and respond to this by activating protease inhibitors.

It would be interesting to investigate the role of nitric oxide; it reduces hydrogen peroxide in-turn repressing \textit{pin2} expression. Do the caterpillars showing lower levels of induction have more nitric oxide in their saliva? It would also be interesting to perform a dose-response model for each species of larvae to investigate how saliva dose-response affects gene induction. Ten caterpillars of saliva per plant were used in the saliva experiment. A model has been done with \textit{Helicoverpa ze}a and has shown a bell-curve response indicating that five caterpillars of saliva instead of ten caterpillars of saliva is more effective in inducing a response (personal communication, Michelle Peiffer.)

No systemic effect was observed in the systemic experiment. The values obtained were very low and variable. The lack of systemic induction observed may relate to the relatively small amount of mechanical wounding involved as well as the tomato cultivar used. The systemic leaves used for the experiment were from an experiment performed in October, the time of year might also play a role in the low levels of induction seen. The treatments were conducted for 24 hours; if the treatments were applied for 36 or 48 hours it may induce a better response.

Glucose oxidase was found to be present in \textit{H. virescens}, \textit{H. ze}a and \textit{S. frugiperda}. All these larvae are fruit-feeding lepidopterans of family Noctuidae. The presence of glucose oxidase
may be attributed to their fruit-feeding eating habits. I was also able to collect saliva from these larvae, they may have larger spinnerets better equipped to secrete saliva and therefore able to secrete more glucose oxidase leading them to produce more in their labial glands.

The results from the preference studies shown that the larvae prefer consuming induced leaf tissue, indicating there is something else playing a role in insect feeding. The results may also indicate that insects cannot taste the protease inhibitors and therefore continue to feed on induced leaf tissue. There may be a feedback mechanism that triggers increased feeding so that the larvae can overcome the adverse effects of the protease inhibitors. The specificity of herbivore-induced responses is still an important area of research and my results indicate that saliva plays a role in determining specificity.
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