Local vs. systemic modifications of soybean (Glycine max) defense signals in response to soybean aphid (Aphis glycines Matsumura) infestation

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Local vs. systemic modifications of soybean (Glycine max) defense signals in response to soybean aphid (Aphis glycines Matsumura) infestation

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of study committee:

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Iowa State University
Ames, Iowa
2015

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DEDICATION

I dedicate this thesis to my family, who have sacrificed and endured so much in a poverty-stricken country so that I may have the opportunity at a better life. I dedicate the works in my past and in my present, as well as the success in my future, to their love, support, and drive. Thank you for the life and love you have given me!
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CHAPTER ONE

INTRODUCTION

The Soybean

The soybean (Glycine max (L.) Merr.) is among the most important crops in the world. It represents 56 percent of the world’s oilseed production, with 9.4 million metric tons being used in oil products, 39.1 million metric tons being used in livestock feed, and 6.6 billion liters used in biodiesel in the United States. In total, soybean production reached a high of 108 million metric tons and a value of $40 billion in 2014 in the United States alone (Soystats, 2014).

The popularity of this plant as a cash crop comes from its versatility in multiple industrial settings. The soybean seed is high in oil content, and this oil may be extracted for uses in nutrition, livestock feed, and biofuel (Soystats, 2014). For example, soy lecithin is harvested as an important component in fish nutrition. Its properties as a bio-surfactant are important in emulsifying fats essential to fish diets (Anicic et al., 2013). Soybean biodiesel is also an important and inexpensive replacement for the fossil fuels it displaces as it provides 93% more energy than is needed for its production (Hill et al., 2006). Soybeans contain remarkably high levels of polyunsaturated fatty acids (PUFAs) which are essential for most mammalian diets (Jiao and Zhang, 2013). Soybean, like most plants, can synthesize a wide range of PUFAs, including oleic acid (18:1, Δ9), linoleic acid (18:2, Δ9,12), and linolenic acid (18:3, Δ9,12, 15). These 18 carbon fatty acids and their metabolic products are important in structural determination of mammalian cells as well as improvement of cardiovascular health; however, as 18 carbon polyunsaturated fatty acids cannot be synthesized in mammals, soybean consumption is well promoted in human diets. Unfortunately, excessive intake of ω-6 fatty acids (18:2 and its products) and deficient intake of ω-3 fatty acids
(18:3 and its products) has shown to be related to cardiovascular diseases, neural diseases, and inflammatory disorders (Jiao and Zhang, 2013). Consequently, researchers have worked to develop breeds of soybean that contain inherently low levels of \( \omega-6 \) fatty acids.

With such extensive production of soybean in the United States, research advocating the crop’s survival and yield is abundant. However, among the many threatening and important pests and pathogens that the soybean faces, the soybean aphid (\textit{Aphis glycines} Matsumura), remains to be one of the most threatening, and over the years, research concerning this pest has begun to gain momentum.

**Soybean Aphids**

The soybean aphid is a native of Asia and was first found in the Midwestern United States during the year 2000, and by the end of that year, its occurrence had spread to other regions of the country (Hartman et al., 2001). Soybean aphids extract sugars and essential amino acids from the phloem sap of soybean, effectively diverting important nutrients and photosynthates from the plant and ultimately resulting in annual yield losses of up to 50% (Studham, 2010; Ragsdale et al., 2007). Soybean aphids, like other species of aphids, are phloem feeders, and damage caused by aphid colonization includes leaf stunting and inhibition of overall plant growth (Li et al., 2008). Aphids typically feed by inserting feeding appendages called stylets through the epidermis, mesophyll, and parenchyma tissue layers to reach the phloem sap (Morkunas et al., 2011). In addition to sapping important nutrients from their hosts, soybean aphids can also transmit viruses from plant to plant, such as the soybean mosaic virus (Hartman et al. 2001).

The relative abundance and survival of the soybean aphids partly depends on its complex life cycle. During the warmer spring and summer months, soybean aphids typically prefer soybeans as their
primary hosts, producing live young at high rates. During the late autumn months, soybean aphids emigrate to the common buckthorn (*Rhamnus cathartica* L.) and survive the winter months by producing overwintering eggs (Ragsdale, 2004). In addition to its complex life cycle, soybean aphids have also been shown to effectively evade host immune responses, prolonging their survival on their hosts and further exacerbating the damage done.

For economies that rely on soybean as a staple crop, the implications are devastating if the threat of the soybean aphid is left uncontrolled. As such, researchers worked to introduce the first varieties of soybean that are resistant to soybean aphids. These varieties contained a, aphid resistance gene, *Rag1* (Ragsdale et al., 2011). However, even with the introduction of this new variety of soybean, one of the more prevailing methods to control soybean aphid infestation remained to be the use of insecticides, which is often not environmentally friendly and can also increase the production costs of soybean (Kanobe, 2012). As a result, much ongoing research has been dedicated to identifying, characterizing, and manipulating plant defense responses to the soybean aphid.

**LITERATURE REVIEW**

**Pathways involved in plant defense responses**

Being almost entirely sessile, most plants have developed complex mechanisms to defend against various types of stresses (Wu et al., 2011). For example, in response to herbivorous insects plants may produce volatiles that attract natural predators to those herbivores or induce the production of chemical and physical barriers against pathogens (Mello and Silva-Filho, 2002). These physical barriers include specific compounds such as callose deposits in the cell wall at the site of fungal infection in *Arabidopsis thaliana* (Nishimura et al, 2003).
In the case of infestation by chewing insects such as caterpillars, resistance is thought to be conferred by the production of chemical barriers such as proteinase inhibitors (PIs), which is elicited not only by insect infestation, but also by mechanical wounding (Accamando and Cronin, 2012). The most common PIs found are inhibitors of trypsin and chymotrypsin (Casaretto et al., 2003). These PIs can work against a wide variety of herbivores as they inhibit the basal activity of protein digestion, forcing the attacker to over-secrete native proteases to overcome the inhibitory effects of PIs and resulting in death or stunting of growth (Broadway and Duffey, 1985). In the case of aphids and barley, the induction of several PIs has been shown to be upregulated upon aphid feeding, although their role in defense against aphids is not known (Casaretto et al., 2003).

The induction of these defense mechanisms is regulated through a system of pathways including the jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) pathways. Both the SA and ET pathways are activated upon pathogenesis, and both induce defense genes on activation (Thaler et al., 2002; Lorenzo et al., 2002). However, the pathway of most interest in terms of insect infestation is the jasmonate pathway, which uses jasmonic acid (JA) as the regulatory phytohormone (Thaler et al., 2004). Jasmonic acid is an oxylipin that is synthesized in the chloroplasts and peroxisomes via the octadecanoid pathway (Wang and Wu, 2013). JA and its derivatives primarily function to regulate both plant growth and defense, and in the case of insect or pathogen attack, JA accumulates directly at the site of infestation (Liu et al., 2010; Wang and Wu, 2013). The production of JA is initiated by herbivory or general mechanical wounding and has been show to directly induce the production of PIs in *Arabidopsis* (Farmer and Ryan, 1992; Turner et al., 2002; McConn et al., 1997). Although most of JA signaling is referred to as JA or the jasmonates, the induction of PIs and other defense signals by JA signaling most likely occurs via its bioactive conjugate with L-isoleucine, JA-Ile, rather than the non-conjugated JA (Svobodad and Boland,
2010). Exogenous application of JA has been shown to replicate the same effects that wounding has, including induction of PIs, and mutants defective in JA biosynthesis or perception have been shown to have greatly reduced resistance to pathogens and insects. These findings strongly reinforce JA as one of the major phytohormones in plant defense (Howe, 2001).

Abscisic acid (ABA) is known as an essential phytohormone in responses to certain stresses. However, whereas JA responds mostly to herbivore, pathogen, and disease related stresses, ABA responds mostly to abiotic stresses such as changes in hydration, temperature, and salinity (Mauch-Mani and Mauch, 2005; Moons et al., 1997). At first glance, the nature of these two pathways appear to act completely independent of one another, given the radically different types of stresses that trigger each phytohormonal signaling. Upon closer examination, however, the ABA and JA pathways have been shown to have significant cross-talk between them (Thaler et al., 2004; Mauch-Mani and Mauch, 2005). These pathways have been shown to be both synergistic and antagonistic to one another, depending on the conditions (Moons et al., 1997). The antagonism in this case may be an upregulation of one and a downregulation of the other and also a way for plants to conserve resources by preventing the production of proteins or products of both pathways (Anderson et al., 2004). Considering how JA is considered as a major player in defense against pathogens, ABA has been often considered to be a negative regulator of disease and pathogen resistance, as exogenous application of ABA in Arabidopsis has correlated with susceptibility to pathogenesis and decreased levels of ABA by inhibition of ABA biosynthesis correlated with increased resistance to pathogenesis (Mauch-Mani and Mauch, 2005).

Until recently, our understanding of ABA signaling in response to biotic stresses was rudimentary. As such, interactions between the ABA and JA pathways are complex and poorly characterized.
However, the effect that both pathways together have on biotic stresses such as aphid infestation is one of importance and cannot be ignored.

The role of fatty acids in plant defense

In addition to being important as structural components in cellular membranes and metabolism in nearly all forms of life, fatty acids and lipids in general can act as defense signal precursors in soybean and other plants (Wang, 2004; Shah, 2005).

Oxylipins such as JA regulate many defense and growth pathways in plants and are derived from the catabolism of polyunsaturated fatty acids (PUFAs), most notably linoleic and linolenic acids (Creelman and Mulpuri, 2002). It has been proposed that upon wounding, the first step of oxylipin biosynthesis is initiated by release of a membrane linolenic acid that goes through subsequent downstream reactions involving lipoxygenases and α-dioxygenases (Farmer and Ryan, 1992; Louis and Shah, 2013; Wang and Wu, 2013). In the case of JA biosynthesis, linolenic acid is released from plastidal membranes and is then converted to 13-hydroperoxylinolenic acid (13-HPLA) by lipoxygenase, which is then dehydrated by allene oxide synthase (AOS) to form an unstable epoxide, 12-13-epoxy-octadecatrienoic acid. Allene oxide cyclase (AOC) then cyclizes this unstable compound to form 12-oxo-phyto-dienoic acid (OPDA). JA is then produced after reduction of OPDA by OPDA reductase and three rounds of beta-oxidation (Creelman and Mulpuri, 2002; Wang and Wu, 2013).

The main PUFAs in some plants including soybean are linoleic (18:2) and linolenic acids (18:3), while other plants contain hexadecadienoic acid (16:2) and hexadecatrienoic acid (16:3) instead. The production of linoleic and linolenic acids begins with palmitate elongation into stearate by a ketoacyl ACP synthase (KAS II). Stearate is then desaturated to form oleate. Oleate may then be
desaturated to linoleic acid via two pathways. In the microsome, oleate desaturation is mainly controlled by a fatty acid desaturase 2 (FAD2) and in the chloroplast, desaturation is controlled by a fatty desaturase 6 (FAD6). Linoleic acid is finally desaturated by FAD3 in the microsome and FAD7 and FAD8 in the chloroplast to produce linolenic acid (Hildebrand, 2011). In developing seeds, the microsomal pathway predominates while in leaves, the plastidal pathway predominates (Yadav et al., 1993). In addition to their importance as direct precursors to oxylipins, fatty acids are also important in protection against cold temperatures in early growth stages (Iba, 2002; Upchurch, 2008). At low temperatures, the membranes of plant cells and plastids become more gel-like and less fluid to reduce leakage of essential nutrients (Upchurch, 2008). PUFAs are especially important in the arrangement of plastidal membrane lipids under cold conditions. Mutants with defective FAD6 and FAD5 activity resulted in accumulation of monounsaturated lipids such as 16:1 and 18:1 and decreased levels of 18:2 or 18:3. These mutants showed less tolerance to cold stresses in the early growth stages than the wild type (Iba, 2002).

Perhaps as important is the role of these fatty acids in defense against pathogens and insects. In Arabidopsis, SSI2 encodes for a stearoyl-ACP desaturase that catalyzes the conversion of stearate (18:0) to oleate (18:1). An Arabidopsis ssi2 mutant has shown to have high accumulation of 18:0 and reduced levels of 18:1. In addition to changes in these fatty acid levels, there was also constitutive repression of the JA signaling pathway (Kachroo et al., 2001; Kachroo et al., 2003b). Another Arabidopsis mutant, fad3-2 fad7-2 fad8, is a triple mutant containing three defective fatty acid desaturase genes. This mutant exhibits low levels of 18:3 and low levels of JA under both wounding and non-wounding conditions. The decreased levels of linolenic acid and consequently JA resulted in the mutant being highly susceptible to feeding by Bradysia impatiens, a species of gnats (Kanobe, 2012; McConn and Browse, 1996). These results suggest that fatty acids, even
ones significantly upstream of oxylipin biosynthesis such as 18:0 and 18:1, are essential in maintaining JA related defense responses.

OBJECTIVES, JUSTIFICATION, AND SIGNIFICANCE

Since plants lack the motility that other organisms have to defend themselves, they have, over the course of evolution, developed diverse defenses against their more mobile enemies and undesirable environment conditions. These defenses present themselves in a complex system of phytohormonal signaling that can affect one another in inhibitory or excitatory ways. These pathways are essential in maintaining plant health and proliferation and as such, researchers have devoted countless efforts to better understand plant defense. In soybean, the major pathways contributing to plant defense against pathogens and insects are the JA, ethylene (ET), and salicylic (SA) signaling pathways, but recent studies have suggested that the ABA signaling pathway also plays a role in this respect (Li et al., 2008, Loake and Grant, 2007). In the case of soybean-aphid interactions, the pathways of highest interest to us are the JA and ABA pathways, which taken alone, control immunity against pathogenesis and abiotic stresses, respectively, and can together influence defense against pests and insects such as the soybean aphid. For example, ABA responsive genes were found to be induced by aphid infestation, and this induction has been hypothesized as a decoy response to suppresses defenses such as JA (Studham and MacIntosh, 2013).

There is now significant evidence of the role of fatty acids in these defenses (Kachroo and Kachroo, 2009). Linolenic acid is deemed as the precursor of the oxylipin pathway that produces the influential phytohormone, JA. Much research has been done to characterize the intermediates in this pathway as well as the precursor fatty acids to the production of linolenic acid. We have
evidence of a consistent and characteristic change in the levels of these precursor fatty acids, namely 16:0, 18:2, and 18:3, upon aphid infestation, and this evidence suggests that the changes in these fatty acids consequently affect plant fitness by dismantling defenses related to a JA response (Kanobe, 2012; Studham and MacIntosh, 2013). The relationship between soybean defenses in response to aphid infestation remains complex and not fully understood. However, the majority of the research done on this relationship has been focused on local responses, while the effect that aphids have on systemic responses are less well characterized. We are quite confident now of the changes to soybean fatty acid levels that soybean aphid infestation induces at a local level, and while there is evidence that soybean aphids increase susceptibility to other pests such as nematodes (McCarville et al., 2012), we remain unaware of the direct consequences soybean aphid impose on soybean induced defense responses.

What changes do aphids effect on fatty acid content and defense gene expression on a systemic level? We have worked to expand these results and explore the effects that aphids have on systemic resistance. More specifically, we studied the changes in three fatty acids, palmitic, linoleic, and linolenic acids, during aphid infestation at regions distal of the site of infestation. The importance of these fatty acids in plant defense is now agreed upon. Therefore, we also included in our study the changes in expression of several genes involved in defense pathways known to be induced by aphid feeding. We also performed an extensive lipidomics analysis to identify other changes triggered by aphids on lipid metabolism. With these results, we hope to better understand soybean local and systemic signaling in response to soybean aphids.
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CHAPTER TWO

LOCAL AND SYSTEMIC APHID-INDUCED EFFECTS ON FATTY ACID COMPOSITION AND PHYTOHORMONAL SIGNALING IN SOYBEAN

Abstract

Soybean aphids, *Aphis glycines*, are a specialized phloem-feeding pest of soybeans. They are known to employ different mechanisms to bypass plant defenses and reduce soybean plant yields of up to 50% a year. Once attacked or sensing distress, soybeans induce the oxylipin pathway, a biosynthetic pathway that produces key plant hormones. Among these hormones is jasmonic acid (JA), which is produced from linolenic acid (18:3). Jasmonic acid can signal the production of protease inhibitors as one of the products that obstructs the attackers’ ability to digest protein. Previous studies show that levels of three key fatty acid precursors (palmitic, 16:0; linoleic, 18:2; linolenic, 18:3) to JA biosynthesis are changed by aphids. We are interested in determining if the change in these fatty acid levels induced by soybean aphids is only a localized response or whether the changes are also triggered systemically. We found that accumulation of 16:0 and a decrease in levels of 18:3 only occurs at the local site of infestation. We also studied the expression levels of *FAD6*, a gene encoding for a fatty acid desaturase catalyzing the dehydration of oleate (18:1) to 18:2, and found that *FAD6* expression levels are also decreased only locally. In addition to the oxylipin pathway, we are also interested in the abscisic acid (ABA) pathway, which is involved in pathogen and abiotic induced stress and is proposed to be antagonistic to the JA pathway in soybean. We used expression of the *SCOF-1* gene as a marker for ABA signaling, and found *SCOF-1* to be induced both locally and systemically in the presence of aphids.
Introduction

The soybean aphid is one of the most important pests of soybean, with the potential to reduce soybean yields of up to 50% a year (Ragsdale et al., 2007). These aphids are specialized phloem feeders of soybean and were discovered in the United States at the beginning of the year 2000 (Ragsdale et al., 2004). Soybean aphids cause plant stunting, leaf distortion, and reduced pod set in addition to deteriorating overall plant health (Li et al., 2008). Soybean aphids and other species of aphids typically feed by inserting their stylets into several tissue layers to reach the phloem, where they sap essential nutrients from their hosts or transmit harmful viruses from plant to plant (Morkunas et al., 2011; Studham, 2010; Hartman et al., 2001). The most common method of aphid management has been the use of insecticides; however, this method is not only costly, but also harmful to the environment. In light of this, innate plant resistance has been shown to be a better alternative to insecticides (Enders et al., 2014).

Soybean, among other plants, has evolved mechanisms to defend themselves against a wide range of stresses, both biotic and abiotic. These mechanisms are mostly manifested in the form of interlinking defense pathways. The jasmonic acid (JA) pathway participates mainly in defense against diseases and other biotic stresses, while in the case of abiotic stresses such as drought, salinity, and cold temperatures, the abscisic (ABA) pathway is favored (Mauch-Mani and Mauch, 2005; Roychoudhury et al., 2013). In addition to defending against biotic stresses, however, JA can also act as a growth regulator by influencing leaf abscission and senescence (Hays et al., 1999). Taken together, JA and ABA can affect each other in both additive and subtractive manners. In rice roots, exogenous application of JA has been shown to reduce the expression levels of ABA sensitive genes (Moons et al., 1997). However, in the case of mechanical damage, such as wounding by insect feeding, both JA and ABA respond by inducing the expression of proteinase
inhibitor (PIN) genes in *Arabidopsis* (Dammann et al., 1996). Proteinase inhibitors are a form of a direct line of defense against herbivores by interfering with the attackers’ digestive abilities (Green and Ryan, 1972). Mechanical damage is hypothesized to induce the ABA pathway, which continues in downstream reactions to activate JA biosynthesis and finally induce PIN expression (Peña-Cortés et al., 1995). Although both general mechanical wounding and insect wounding induce PIN expression, it has been shown that insect feeding creates a stronger PIN induction than mechanical wounding, which may be attributed to the plant’s ability to recognize molecular secretions made by the insect upon feeding (Walker-Simmons et al., 1984; Roda et al., 2004). Other studies suggest that PIN induction by wounding happens via signal transduction between ABA and JA and that this transduction occurs somewhere downstream of ABA activation (Peña-Cortés et al., 1993). Whereas this relationship is a synergistic one in *Arabidopsis* and tomato, recent research has shown that induction of the ABA pathway may lead to suppression of the JA pathway and consequently reduction in PIN expression and other JA-dependent defenses in soybean. Studham and MacIntosh (2013) carried out an analysis of transcriptome changes triggered by aphid feeding in soybean, at 1 day and 7 days after colonization. In the early stage, aphid feeding induced expression of JA biosynthetic genes and also increased the expression of JA-regulated markers, indicating that the JA pathway was functional. However, at the later time point the JA-regulated expression disappeared even though expression of JA biosynthesis genes was 5-fold higher than at the early time point. This apparent block in JA signaling was paralleled by a very significant increase in the expression of ABA biosynthetic genes and ABA-regulated genes. These observations led to the hypothesis that long-term aphid feeding triggers an ABA-dependent response able to block effective JA-regulated defenses, and in that way increase susceptibility (Studham and MacIntosh, 2013). Recent experiments have shown that ABA
pretreatments can block JA-dependent increases in gene expression in response to wounding in soybean, supporting the antagonism between ABA and JA signaling pathways in this plant (Hohenstein and MacIntosh, unpublished). This antagonistic relationship between ABA and JA could account for soybeans’ relatively high susceptibility to soybean aphids.

In addition to phytohormones such as JA and ABA, fatty acids are also essential in defense against herbivores and insects such as the soybean aphid. Linolenic acid (18:3, Δ9, 12, 15) is a common constituent in membrane lipids and also serves as the substrate to the biosynthetic oxylipin pathway that produces JA as one of the end products (Kong et al., 2005). Exogenous application of linolenic acid resulted in higher induction of PIN expression than wounding, suggesting that this compound is readily converted into JA. Other fatty acids, namely palmitic and linoleic acids, did not produce as strong of a PIN induction as linolenic acid, implying that linolenic acid is a central compound in JA derived defense (Farmer and Ryan, 1992). Recent studies have shown that upon aphid infestation of soybean, there is an accumulation of palmitic acid and a reduction in levels of linoleic and linolenic acids in local tissue. Consequently, low levels of proteinase inhibitor II (PIN2) expression is also observed, suggesting that aphids are effectively dismantling JA defenses by affecting the levels of JA biosynthetic precursor fatty acids (Kanobe, 2012).

Kanobe (2012) presented a significant analysis of fatty acid changes associated with aphid infestation in soybean. However, these efforts have been mostly made to characterize the role of fatty acids only in the local tissue (at the site of aphid infestation). Yet, analysis of plant-mediated interactions between different biotypes of soybean aphids or the insect and other soybean pests like the soybean cyst nematode indicated that aphid feeding produces systemic effects on soybean plants (McCarville et al, 2012; Varenhorst et al, 2015). Still, little is known of the changes that aphids effect on these fatty acids at systemic tissue or the effects that aphids induce on systemic
signaling as a whole. As with the immune systems of many other forms of life, plants can become more resistant to a particular breed of pathogenic or insect threat by recognizing the changes made within the plant during the first attack (Karban and Myers, 1989). A form of plant resistance, called induced systemic resistance, constitutes a way for plants to systemically increase their defenses and fitness against a small range of pathogens via a network of systemic signals (Pieterse et al., 2000). However, the mechanisms that allow plants to increase their systemic resistance in this way may not be the same for insect herbivores as they are for pathogens (Bostock, 2005). The significant effects that soybean aphids have on total soybean fitness is an indication of either the attacker’s ability to successfully evade systemic defenses or of the plant’s inability to properly mount systemic defenses against soybean aphids.

We investigated the changes in the fatty acids palmitic, linoleic, and linolenic acids on tissue distal of the site of aphid feeding to study the implications on systemic defense via the JA pathway. In addition to this, we also studied the systemic effects of aphid infestation by examining SCOF-1, an ABA induced transcription factor (Kim et al., 2011), previously shown to be regulated by aphid feeding in local tissues (Studham and MacIntosh, 2013).

**Results**

*Effect of aphid infestation on fatty acid content in local and systemic tissue*

We determined the effect of aphid infestation on local and systemic fatty acid composition on a susceptible variety of soybean under controlled conditions and environment. The results are based on free fatty acid content as percentages of the total amount of fatty acids 7 days after aphid infestation. Aphids were caged to limit their movement to a small portion of the infested leaf. Samples were taken after 7 days of feeding from the caged portion of the infested leaf (region A),
a non-infested section of the same leaf (region B) and a non-infested, younger leaf in the same plant (region C), as shown in Figure 1. Control samples were taken from the same sections of plants with cages but without aphids. In region A, the local tissue, we observed an increase of over 60% palmitic acid content when compared to the control (fig. 2A), which is deemed significant by statistical analysis (P-value<0.05). Linoleic acid (fig. 2B), however, saw no significant changes when compared to the control while linolenic acid (fig. 2C) showed a 30% decrease in content, which is also significant by statistical analysis. Neither region B, the non-infested tissue of the local leaf, or region C, the systemic leaf (fig. 2), showed significant changes in either palmitic, linoleic, or linolenic acid content when compared to the control.

**Effect of aphid infestation on FAD6 and SCOF-1 expression levels**

Since Kanobe (2012) data showed significant reduction of linoleic and linolenic acid contents on aphid infested tissue, he proposed that aphids block the conversion from monounsaturated FA to polyunsaturated FA. Thus, we investigated the effect that aphids have on FAD6 (fig. 3) expression levels, as FAD6 encodes for a chloroplast fatty acid desaturase that catalyzes the desaturation of oleic acid to linoleic acid. For this analysis we used the same experimental setup as used for fatty acid analysis. In the local tissue (region A in fig. 1), we observed a significant reduction in FAD6 expression 7 days after aphid infestation. In the systemic tissues (samples B and C), however, we observed no significant changes in expression levels compared to the control.

In addition to FAD6, we also tested SCOF-1 (fig. 4) as a marker gene for ABA signaling. This cold stress inducible gene is activated by ABA, and based on microarray experiments, it is also strongly induced locally by aphid feeding (Studham and MacIntosh, 2013). Again, the same experimental setup, as described in figure 1, was used. We confirmed strong induction of SCOF-
in local tissues, even though our differences are not significant, likely due to large difference between experiments, which increased overall variability. We also found SCOFL expression levels to be significantly increased by aphid infestation in systemic tissue, both in the non-infested section of the local leaf and in the systemic leaf. Thus, while the effect of aphid feeding on fatty acids is exerted only on the site of feeding, the induction of the ABA pathway seems to be induced both locally and systemically by soybean aphids.

Discussion

Being one of the first lines of defense against enemies such as the soybean aphid, the JA pathway is central to research improving soybean fitness against such pests. As the precursor compounds to this pathway, fatty acids are also equally important in soybean defense research. Not only are fatty acids vital components of cellular membranes, they also play roles in storing energy, acting as substrates in larger metabolic processes, and most importantly, free fatty acids can act as cellular signals for defense (Wang, 2004; Shah, 2005). This prompted research on the effect soybean aphids have on fatty acid content, both locally (Kanobe, 2012) and systemically.

In this research, we found aphid infestation to significantly reduce the levels of linolenic acid and significantly increase the levels of palmitic acid in the local tissue. These changes in both fatty acids have been studied and documented prior to this study by Kanobe (2012). Since the production of polyunsaturated fatty acids (PUFAs) such as linoleic and linolenic acids begin with the elongation of palmitate to stearate (Thelen and Ohlrogge, 2001), with the accumulation of palmitic acid and the reduction of linolenic acid, we suspect that aphid infestation modifies an important step in the conversion of palmitic acid to linolenic acid. We found no significant changes in the levels of other fatty acids between palmitic acid and linolenic acid, suggesting that the
accumulation of palmitic acid may be the main cause for the reduction of linolenic acid. Linolenic acid serves as the main substrate for the branching oxylipin pathway and is fed into it from plastidal membranes upon wounding (Creelman and Mulpuri, 2002). With JA being a product of a branch of the oxylipin pathway, the reduction of linolenic acid levels is suspected to negatively affect the production of JA. Kanobe (2012) showed that low levels of linolenic acid correlated with low levels of PIN2 expression. The reduction of linolenic acid levels in this way allows soybean aphids to effectively improve their own fitness and evade soybean immune responses when feeding. The changes in palmitic and linolenic acids are observed only at a very local level, however. We observed no changes in systemic tissue, even if that systemic tissue is only centimeters away from the site of infestation (region B). This suggests that while regions distal of the site of infestation could be not any more susceptible to aphid infestation compared to the control, they are effectively left unprimed to and seemingly unaware of aphid colonization. The systemic regions B and C probably see unchanged levels of JA induction, and most likely PIN expression. Soybean aphids, in this way, have effectively evaded host defenses directly where they feed and done so without alerting other regions of the plant to induce systemic defenses.

Studies on oleic acid in soybean seed by Kanobe (2012) showed the levels of oleic acid to be significantly increased and studies on leaf tissue showed the levels of linoleic acid to be significantly decreased in the presence of soybean aphids. This prompted us to examine the expression levels of one of the major enzymes in the metabolism of these fatty acids to determine if there is a correlation with oleic acid changes in leaves. The prime candidate was an omega-6 desaturase gene, FAD6, which introduces a double bond at the sixth carbon position of oleic acid to form linoleic acid (Chi et al., 2011). We observed a significant reduction of FAD6 expression levels in the local tissue and no change in the systemic tissue. This change, however, does not
correlate well with our observations of levels of oleic and linoleic acid content; neither fatty acid is significantly changed in the presence of aphids. A reduction in FAD6 expression would intuitively correlate with increased levels of oleic acid. However, Arabidopsis fad6 mutants show a reduction in linolenic acid without an effect on linoleic levels (Kachroo et al, 2003). On the other hand, the Arabidopsis fad6 mutant accumulates oleate and not palmitate. The possibility of a negative feedback loop could instead account for the results observed in soybean. Accumulation of oleic acid above wild type levels may prove to be toxic to soybean, resulting in a negative feedback response that accumulates palmitic acid instead. Thus, reduction of FAD6 expression in soybean could serve to keep stearic, oleic, and linoleic acids levels too low to sufficiently create linolenic acid. No changes are observed in systemic expression levels of FAD6, which reinforces the lack of changes observed within fatty acid content in the same regions.

The ABA pathway has been shown to interact with the JA pathway in synergistic ways in defense against insects in some plants such as Arabidopsis and tomato (Thaler et al., 2004; Mauch-Mani and Mauch, 2005; Damman et al., 1996; Peña-Cortés et al., 1995). However previous studies have shown that induction of ABA responses significantly reduce expression of PIN in soybean (Hohenstein and MacIntosh, unpublished), which prompted us to explore systemic changes in ABA signaling. By using SCOF-1 as a marker for ABA, we observed significant increases in SCOF-1 expression systemically. While the data suggests that local SCOF-1 expression is increased by aphids, the increase is not significant. This lack of significance is most likely due to variance between experimental data that we compiled together. Our results suggest that the ABA pathway is then activated systemically. The induction of the ABA pathway in this way may account for the repression or lack of activation of the JA pathway in systemic tissues. The ABA pathway, therefore, serves as a decoy pathway of sorts that soybean aphids activate in order to
avoid induced host immune responses. However, the repression of JA by ABA is not well characterized. This repression most likely happens downstream of linolenic acid biosynthesis as systemic fatty acid content remains unchanged by soybean aphids.

The issue of soybean aphid evasion of soybean immune defenses is twofold; repression of a local JA signal by changing fatty acid biosynthesis to dismantle defenses at the site of infestation and activation of a decoy ABA pathway to suppress successful priming of systemic defense signals. The pair of these issues in unison may be one of the main causes of soybean susceptibility to soybean aphids. There has been an increasing amount of efforts to research and characterize the interactions between soybean and soybean aphids (Tilmon et al., 2011; Li et al., 2008; Kanobe, 2012; Studham and MacIntosh, 2013), however, further studies must be done to better understand induced soybean defenses to the soybean aphid and induced susceptibility triggered by the insect.

Materials and Methods

Experimental setup and design

This experiment was carried out in growth chambers at a constant 25°C and 16 hours of sunlight at the Iowa State University Agronomy Hall. Typically, 18 pots were planted with 2 SD01-76R (susceptible) seeds in each plot. At around growth stage V1 (Fehr and Caviness, 1977) the healthier or larger plant in each pot was kept for the remainder of the experiment while the other plant was uprooted and discarded. When most of the plants have reached growth stage V2 20 young aphids were placed onto half of the middle leaflet of the V1 trifoliate of 9 of the plants and caged using a leaf cage. These 9 plants served as the experimental group while the other group of 9 plants, treated identically but without aphids, served as the control group. The region of infestation was labeled as region “A,” the local region. The region directly adjacent to region A on the same leaflet was
labeled as region “B,” which is considered as systemic tissue as it lacked any aphids. The middle leaflet of the V2 trifoliate was then labeled as region “C,” also systemic tissue. Aphids on region A were caged with leaf cages and were allowed to colonize for 1 week. Regions B and C, as well as all regions on the control plants, were also covered with leaf cages to reduce any variability that these cages might introduce (fig. 1).

Tissue samples from each region were collected 7 days after infestation. At the time of collection, aphids were brushed off with a soft brush at region A. Regions B and C, as well as all the tissues of the control plants, were also brushed to avoid any variability introduced by this treatment. Tissue was collected in threes and pooled together; therefore, each sample is a collection of 3 biological replicates. Collected tissue was quickly wrapped in pre-frozen and labeled aluminum foil and temporarily stored in liquid nitrogen. This tissue was then kept at -80°C until further processing.

**Fatty acid extraction and analysis**

Prior to extraction, each sample was ground under liquid nitrogen in mortar and pestles and transferred to plastic tubes for storage. Fatty acid extraction was done following a slightly modified protocol described by Kanobe (2012). To begin, 100mg of ground tissue was placed in 10ml glass tubes, 1ml of hexanes was added, and then the tubes were vortexed to homogenize the mixture. After all samples were homogenized, the samples were left to stand overnight covered by aluminum foil to reduce exposure to light. Then 200μl of the oil-hexane mixture were transferred into gas chromatography (GC) vials. To begin transmethylation of fatty acids, 500μl of sodium methoxide was added and the reaction was left to run for 1.5 hours. 150μl of water were then added to stop the reaction and the GC vials were filled to the neck with hexane. The samples were then
run with GC-flame ionization detection (FID). The fatty acid content was given as a percentage of the total amount of fatty acids.

**RNA extraction and gene expression analysis**

RNA was isolated from ground tissue using the Tri Reagent Solution (Ambion). This was followed by cDNA synthesis with the qScript cDNA Synthesis Kit (Quanta Biosciences). Finally qPCR samples were prepared with the PerfeCta SYBR Green FastMix, Low Rox (Quanta Biosciences). These samples were then run with the Stratagene Mx4000. All procedures followed manufacturer’s instructions.

**Statistical analysis**

This experiment was performed 3 times under the same conditions. For the fatty acid analysis, all raw data from each experiment was compiled, averaged, and analyzed with a Two Sample T-test Assuming Unequal Variances in Excel. For gene expression analysis, data in individual experiments was first normalized to the expression of ubiquitin. Then, data from whole experiments was normalized to minimize variable expression levels by dividing each data point in either the experimental or control group by the average of all data points in that group. The normalized data is then compiled, averaged, and also analyzed with a Two Sample T-test Assuming Unequal Variances in Excel. Significance was determined by a two tailed P-value < 0.05.

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Figure 1: Experimental setup for fatty acid and gene expression analysis. The V1 leaf is divided into two regions A, the local region, and B, the systemic region. Region C is on the V2 leaf and is also the systemic region. 20 aphids were infested at region A and were allowed to colonize for 7 days, at which point samples were collected according to their regions. Aphid movement was restricted within region A by leaf cages, which were also present on regions B and C on both infested and control plants to reduce variability.
**Figure 2: Soybean aphids change local fatty acid composition.** Fatty acid content as percentages of the total amount of fatty acids in local and systemic tissues. Asterisks (*) indicate significant differences (P-value<0.05) between aphid infested (+) and control (-) plants. Leaves were infested with 20 aphids and were left to be colonized for 1 week before samples were collected. Within one week, local levels of 16:0 (A) increased in the presence of aphids while levels of 18:3 (C) decreased. Local levels of 18:2 (B) were left unchanged by aphids. There was no significant change in the level of any fatty acid in systemic tissue.
Figure 3: Soybean aphids significantly modify local levels of \textit{FAD6} expression.

Local (region A) \textit{FAD6} expression is significantly reduced (* = P-value<0.05) in the presence of aphids (+) and is unchanged in systemic tissues (regions B and C) against the control (-). This reduction could account for increased levels of 16:0 via a negative feedback loop under the assumption that 18:1 is toxic to soybean.
**Figure 4:** Soybean aphids significantly modify systemic expression of *SCOF-1*. *SCOF-1* appears to be significantly modified (* = P-value<0.05) only at systemic tissue (regions B and C) in the presence of aphids (+) when compared to the control (−). Although there appears to be a strong induction of *SCOF-1* locally at region A, this induction is not significant most likely due to variations between individual experiments. We expect ABA signaling to follow a similar pattern of systemic induction as *SCOF-1* expression is directly controlled by ABA.
CHAPTER THREE

CHANGES IN SOYBEAN LIPIDOME AND TRANSCRIPTOME IN RESPONSE TO

SOYBEAN APHID INFESTATION

Abstract

Soybean, like many other plants, uses lipids as defense signals when being attacked or sensing distress. Soybean aphids, *Aphis glycines* Matsumura, are one of the major pests of soybean, reducing yields of up to 50% a year. This damage is attributed to the soybean aphids’ ability to successfully evade hosts defenses, namely JA related defenses that is induced after subsequent metabolism of membrane lipids. We have performed a lipidomics analysis on soybean tissues that were infested with soybean aphids for 7 days. We found significant changes in several lipid species including digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), phosphatidyglycerol (PG), lysophosphatidylethanolamine (lysoPE), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA). The changes of highest interest are presented in significant increases of PA and PE, where not only individual species, but also total PA and PE content were affected. To complement the lipidomics analysis, we mined transcriptome data from a previous experiment carried out in the same conditions, looking for changes in expression of genes related to lipid metabolism. Transcriptome data showed an increase in the expression of several lipid metabolism related genes including phospholipase A, phospholipase C, and phospholipase D. We also found several important genes that regulate oxylipins biosynthesis to be upregulated upon aphid feeding. Finally, we found several lipid-binding genes to be induced and some to be repressed by aphid feeding. Together, our results suggest that phospholipids play a role in the soybean response to aphid feeding.
Introduction

The soybean is one of the most versatile and essential crops in many countries around the world today. Its value stems from its nutrition not only for human diets, but also livestock diets as well. Soybeans have a high oil content, and this oil can be extracted for biofuel among other uses. The totality of the soybean’s value sums to approximately $40 billion in the United States (Soystats, 2014). However, an important issue undermines the plant’s value. Soybean aphids, *Aphis glycines* Matsumura, are a specialized class of arthropods that were originally native to Asia (Hartman et al., 2001). With its occurrence in the Midwestern United States during the year 2000, the soybean aphid brought with it soybean yield reductions of up to 50% annually (Ragsdale et al., 2007). Soybean aphids are termed specialized in the sense that they primarily colonize only two different species of plant; soybean during the summer to produce live young and the common buckthorn during the winter to produce eggs (Ragsdale, 2004). With this complex life cycle and the ability to take up nutrients from the phloem of their hosts, soybean aphids have developed mechanisms to avoid soybean defenses, resulting in the extensive yearly yield losses (Morkunas et al., 2011; Li et al., 2008).

Soybean, however, possess many different mechanisms for defense against a wide variety of stresses, including insects such as the soybean aphid. Physical defenses may include callose deposits at lesions created by viruses presumably to isolate the infected area (Kohle et al., 1984; Li et al., 2011). In the case of chemical defenses, a network of cross-talking pathways including the jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) pathways play major roles in resistance against insects and diseases (Mauch-Mani and Mauch, 2005). The abscisic acid (ABA) pathway is another important pathway that primarily deals with abiotic stresses such as cold and salinity, but there is increasing evidence of this pathway’s involvement in defenses against biotic...
stresses as well (Moons et al., 1997; Mauch-Mani and Mauch, 2005). In the case of soybean infestation by the soybean aphid, the JA and ABA pathways remain to be of the most interest. Precursor fatty acids to this pathway are significantly changed by aphids (Kanobe, 2012; see also Chapter 2) and ABA has been shown to be induced and antagonistic to JA signaling during aphid infestation (Hohenstein and MacIntosh, unpublished).

The precursor fatty acids are important not only as precursors to the major JA defense pathway, but are also substituents of lipids in cellular, microsomal, and plastidal membranes (Hildebrand, 2011; Wang, 2004; Shah, 2005). These membrane lipids are then important as signaling molecules in responses to various stresses, and their products formed by phospholipase activity can consequently serve as secondary messengers to many processes. For example, phosphatidylinositol (PI) is a substrate for phospholipase C (PLC), which produces inositol phosphates and has been hypothesized to play a role in not only modifying membrane components but is also involved in a wounding response (Zhai et al., 2011; Mosblech et al., 2008). Mosblech et al. (2008) showed that an inositol 1,4,5-triphosphate (InsP₃) is released upon wounding in wild type Arabidopsis, but in mutants deficient in JA biosynthesis, levels of InsP₃ is unchanged in early time points. These and other findings (Mosblech et al., 2011) support the hypothesis that phosphatidylinositol and its metabolites are essential for JA signaling. Furthermore, in the case of insect attack, Ca²⁺ has been shown to be released from internal compartments by inositol hexakisphosphate (InsP₆). In addition to its role in intracellular signaling, this cation also plays a role in phloem sieve elements occlusion, a main line of defense against phloem feeders (Howe and Jander, 2008; Hung et al., 2004; Will et al., 2013). Phosphatidic acid (PA) is another lipid of high interest, as studies have shown that ABA induces phosphatidic acid release from plasma membranes (Zhang et al., 2004). The release of PA in this way is mediated by phospholipase D
(PLD) activity that hydrolyzes membrane lipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (Zhang et al., 2005; Testerink et al., 2004). Phosphatidic acid has also been shown to positively affect JA signaling, as the precursor fatty acids to the production of JA can be derived from PA (Zhao, 2015). PE is another membrane lipid proposed to be associated with ABA signaling. N-Acylethanolamines (NAEs) can be derived from PEs and have been shown to have a synergistic relationship with ABA-regulated growth processes (Blancaflor et al., 2013).

We obtained a lipid profile (lipidomics analysis) of aphid infested and control leaves in hopes to better understand the relationship between lipid metabolism and aphid infestation. Previous experiments showing increased levels of palmitic acid (16:0) and decreased levels of linolenic acid (18:3) and presumably decreased JA responses (chapter 2) prompted us to examine the entire lipid profile for related changes. We also examined this lipid profile in tandem with microarray results of aphid infested leaves obtained previously to determine any relationships between the changes in lipid content and gene activity of several phospholipases including PLC and PLD. We also obtained the level of expression of several oxylipins biosynthetic and lipid-binding genes.

**Results**

**Changes in soybean lipidome triggered by soybean aphids**

The lipid profiling was performed on SD01-76R (susceptible) soybean leaves that were infested with approximately 20 soybean aphids for 7 days, as well as non-infested leaves that served as the control. The results obtained (table 1) are reported as percentages of total lipid content. In total, 147 species of lipids were detected, but only 85 were selected for downstream statistical analysis to include only those with acceptable coefficients of variation (CoV<0.3). Out of the 147 species detected, a large majority (57%) of these belonged to monogalactosyldiacylglycerols (MGDGs),
with the second most abundant group (30%) being the digalactosyldiacylglycerols (DGDGs). The remaining groups of lipids had lower but detectable percentages of the total lipid content.

Of the galactolipids analyzed, we found 2 species of DGDGs (34:4; 34:3) to be significantly reduced and 1 species of DGDGs (36:6) to be significantly increased. Overall, there was no significant change to the total amount of DGDGs. We also found 2 species of MGDGs (38:5; 38:4) to be significant increased, but the overall MGDG content was unchanged (table 1A).

Of the phospholipids analyzed, 2 species of phosphatidyglycerol (PG) were significantly reduced, and 6 species of PC, 4 species of PI, and 1 species of lysophosphatidylethanolamine (lysoPE) were significantly increased. When looking at total content of these phospholipids, no group was significantly changed by aphid infestation. On the other hand, we found 8 species of PA and 12 species of PE to be significantly increased. The total content of both PA and PE were also significantly increased (table 1).

**Changes in soybean transcriptome by soybean aphids**

The transcriptome results presented in this study are extracted from the microarray performed by Studham and MacIntosh (2013), using a more relaxed cutoff. Table 2 shows significant changes (P-value<0.05; FDR<0.05; fold change ~2 or higher) in many lipid metabolism genes including upregulation of phospholipase A (PLA), PLC, and PLD. The majority of lipid metabolism genes are upregulated (highlighted yellow) with the exception of a few genes that are downregulated (highlighted blue). Of the genes downregulated, a fatty acid desaturase encoding transcript (FAD7/8) is of high interest. FAD7/8 encodes for an omega-3 desaturase that catalyzes the conversion of linoleic acid (18:2) to 18:3, a major substrate to the JA producing oxylipin pathway. Table 3 shows oxylipins biosynthetic genes, which are all shown to be significantly upregulated
upon aphid feeding. Table 4 shows genes corresponding to lipid binding proteins, the majority of which are upregulated. Several genes in this list correspond to lipid binding proteins regulated by Ca\(^{2+}\), or with specificity for phospholipids.

**Discussion**

Lipids are important components in cellular membranes, but now lipids and their metabolites are known to be essential in many signaling processes including defense signaling (Hildebrand, 2011; Zhai et al., 2011; Mosblech et al., 2008). Our lipidomics analysis shows that many membrane lipid species are significantly changed by aphids. Several species of PA are significantly increased (table 1B). PA has been shown to have ties to both the JA pathway as one of the sources of 18:3 that feeds into JA synthesis as well as the ABA pathway, in which PA production has been shown to be induced by ABA (Zhang et al., 2004; Zhao, 2015). This increase in levels of PA correlates well with the increase in PLD activity in our transcriptomic analysis, as PLD is the enzyme responsible for producing PA from hydrolyzing phospholipids (Zhang et al., 2005). Such an increase in PA could signify an increase in both ABA and JA signaling. However, as Hohenstein and MacIntosh (unpublished) have shown that ABA activation is probably antagonistic to JA-regulated responses, it is likely that this increase in PA is due to activation of ABA by a wounding response (Peña-Cortés et al., 1995), and by this assumption, we can further reinforce that aphids block JA-regulated defenses by inducing the ABA pathway in soybean. On the other hand, it is possible that while JA-related responses are being blocked by ABA, the plant is attempting to produce enough JA to overcome this inhibition, resulting in increasing levels of PA to fuel JA biosynthesis. The apparent increase in PC and PE can also be a result of an increased ABA response as PC and PE are both substrates for PLD and sources for PA. However, although ABA is antagonistic to JA, there is still a multitude of oxylipin biosynthetic genes that are upregulated
during aphid feeding (table 3). Although these genes are related to JA biosynthesis, there appears to be no induction in a JA signal 1 week after aphid infestation (Studham and MacIntosh, 2013). Therefore, it is possible that a different branch of the oxylipin pathway that converts linolenic acid into 13-hydroxylinolenic acid and produces green leafy volatiles (chemicals purposed to prime neighboring plants’ defenses) is induced in place of JA biosynthesis (Savchenko et al., 2013; Howe and Jander, 2008) or simply because the production of JA is ineffective in creating a signal.

PE is not only a source for PA but is also a precursor to NAEs, compounds known to have synergistic effects with ABA. Induction of an ABA response resulted in NAE accumulation, and exogenous application of NAEs activated many ABA responsive genes (Blancaflor et al., 2013). Our lipidomics analysis shows significant increases in many species of PEs (table 1A), suggesting that the production of NAEs, and consequently induction of ABA, is also increased. This assumption is in line with the proposed relationship between aphid feeding, ABA, and JA-regulated defenses by Hohenstein and Macintosh (unpublished). The increases in ABA responses by PEs in this way could be another outlet for soybean aphids to repress soybean defense.

PIs have been shown to be important in wounding and JA-related responses (Mosblech et al., 2008; Mosblech et al., 2011). Its derivatives, InsP$_3$ and InsP$_6$, have both been shown to be released by wounding. We see significant increases in several species of PIs by aphid feeding (Table 1B). In this respect, InsP$_6$ is especially important as it is one of the major players in defenses against phloem feeding insects (Hung et al., 2014). Phloem sieve elements occlusion is a method of defense in which sieve cells lining the phloem of plants are essentially closed up to limit the loss of phloem sap (Will et al., 2013). This defense mechanism is controlled by a transient release of Ca$^{2+}$ in the endoplasmic reticulum into the sieve element cytoplasm by InsP$_6$ (Hung et al., 2014). There is not only an increase in levels of PIs but also an increase in levels of PLC (table 2A).
expression, suggesting that inositol phosphates such as InsP$_3$ and InsP$_6$ are transiently produced by aphid infestation. Furthermore, several calcium-regulated lipid-binding genes (table 3) are also upregulated, further reinforcing the induction of phloem sieve element occlusion through the metabolism of PIs as a line of defense against soybean aphids. Unfortunately, the induction of phloem sieve element occlusion alone is not enough to raise the overall resistance of soybean to soybean aphids.

As shown, soybean membrane lipids and several of their metabolites have profound and complex effects in defense against soybean aphids. Lipids such as PA and PE probably interact with ABA to reduce overall susceptibility by repressing JA-related defenses. On the other hand, PIs could be strong mediators of resistance against soybean aphids by effecting physical changes in the phloem.

**Materials and Methods**

*Experimental setup and design*

The plants for this experiment were of SD01-76R (susceptible) soybean variety and were grown in growth chambers kept at a constant 25°C and 16 hours of light at the Iowa State University Agronomy Hall. 20 pots were seeded with 2 seeds each and were watered 3 times a week. At approximately growth stage 1 V1, the healthier plant was selected for and the other was discarded. At approximately growth stage 2 V2, 10 plants were chosen at random and were infested with 20 soybean aphids on the middle leaflet of the V1 trifoliate. The remaining 10 plants served as control plants with no aphid infestation. After the initial infestation, the aphids were left to colonize for exactly 1 week, at which point samples were collected. Samples were collected by cutting across the midvein of the middle V1 leaflet and immediately wrapping the sample in labeled aluminum foil and immersing it in liquid nitrogen. Samples were then stored at -80°C until further extraction.
Lipid extraction and tandem mass spectrometry analysis

The lipid extraction protocol followed is a slightly modified version of the method described by Welti et al., 2002, and the analyses done in this study were performed at the Kansas Lipidomics Research Center (KLRC) Analytical Laboratory. Briefly, the entire V1 half leaflet is immersed in 3ml of isopropanol with 0.01% BHT (butylated hydroxytoluene) that was preheated to 75°C for 15 minutes in Teflon-lined glass tubes. Then, 1.5ml chloroform and 0.6ml water were added, vortexed, and the mixture was shaken at room temperature for 2 hours. The lipid extracts of this step were then transferred to new Teflon-line glass tubes with glass pipettes and were stored under the hood. Next, 4ml of chloroform/methanol (2:1) with 0.01% BHT was added to the initial glass tubes and were shaken for 2 hours. This step was repeated 4 times until the leaves turned white, indicating that the lipids were fully extracted. Finally, all of the extracts were pooled into one and 1ml 1M KCl was added, vortex, and centrifuged at 6000rpm for 2 minutes. The upper phase was discarded and 2ml of water was added to the lower phase. This mixture was again vortexed and centrifuged at 6000rpm for 2 minutes and the upper phase was discarded. The samples were then evaporated under nitrogen gas and were shipped on dry ice to the KLRC Analytical Laboratory. The extracted leaves were then dried for 8 hours in an oven at 100°C and then weighed for their dry weights. The KLRC Analytical Laboratory then performed tandem mass spectrometry on each sample using standards from each lipid head group as described by Welti et al., 2002.

Data processing and statistical analysis

The lipidomics analysis was performed on ten individual samples for each treatment. The lipid profile was returned from KLRC Analytical Laboratory as an Excel spreadsheet with lipid content reported as both percentages of the total lipid content as well as lipid content per dry weight.
Covariance analysis identified those lipid species that could be analyzed further. We used the lipid content reported as percentages of the total as it applied better to our analysis. A Two Sample T-test Assuming Equal Variances comparing the experimental to the control was run in Excel for all lipid species detected, but in this study, we only reported those lipid species that showed significant changes (two tailed P-value < 0.05).

**Transcriptome data**

Gene expression data were extracted from a transcriptome dataset comparing aphid-infested and control plants after 7 days of aphid feeding. The dataset and bioinformatics analysis were described in Studham and MacIntosh (2013). For the current analysis we relaxed the significance (P-value) and false discovery rate (FDR) parameters to make the analysis more inclusive. Only data with P-value<0.05 and FDR<0.05 were extracted, and those with absolute fold change of ~2 or higher are reported. Lipid-related genes were identified by querying individual gene annotations based on GO-terms and BLAST results, using an in-house script followed by manual curation.

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TABLES

Table 1A: Changes in lipid profile of aphid infested leaves. The species of lipids shown in this table have shown significant changes during aphid infestation (p-value < 0.05). Also listed in this table are the averages (Avg.) of each lipid species in both infested and control leaves as percentages of the total lipid content as well as the corresponding standard deviations (STDev.) The response column shows the effect aphids have on a particular lipid species in comparison to the control.

<table>
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<th>Species¹</th>
<th>Aphid infested</th>
<th>Non-aphid infested</th>
<th>P-value</th>
<th>Response</th>
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<td>34:4</td>
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<td>6.518 0.820</td>
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<td>Decrease</td>
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<td>19.100 2.995</td>
<td>16.897 1.714</td>
<td>0.059</td>
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</tr>
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<td>PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32:0</td>
<td>0.010 0.002</td>
<td>0.014 0.004</td>
<td>0.030</td>
<td>Increase</td>
</tr>
<tr>
<td>34:4</td>
<td>0.010 0.002</td>
<td>7.86E-03 1.88E-03</td>
<td>0.013</td>
<td>Increase</td>
</tr>
<tr>
<td>36:6</td>
<td>0.345 0.035</td>
<td>0.299 0.044</td>
<td>0.018</td>
<td>Increase</td>
</tr>
<tr>
<td>38:6</td>
<td>5.53E-03 9.79E-04</td>
<td>4.27E-03 5.39E-04</td>
<td>0.002</td>
<td>Increase</td>
</tr>
<tr>
<td>38:3</td>
<td>0.033 0.003</td>
<td>0.029 0.004</td>
<td>0.018</td>
<td>Increase</td>
</tr>
<tr>
<td>40:3</td>
<td>6.50E-03 9.96E-04</td>
<td>5.42E-03 1.19E-03</td>
<td>0.041</td>
<td>Increase</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36:6</td>
<td>0.118 0.024</td>
<td>0.088 0.018</td>
<td>0.005</td>
<td>Increase</td>
</tr>
<tr>
<td>36:5</td>
<td>0.261 0.052</td>
<td>0.196 0.060</td>
<td>0.017</td>
<td>Increase</td>
</tr>
<tr>
<td>36:4</td>
<td>0.222 0.045</td>
<td>0.168 0.063</td>
<td>0.040</td>
<td>Increase</td>
</tr>
<tr>
<td>36:3</td>
<td>0.158 0.027</td>
<td>0.120 0.030</td>
<td>0.009</td>
<td>Increase</td>
</tr>
<tr>
<td>36:2</td>
<td>0.126 0.023</td>
<td>0.096 0.031</td>
<td>0.027</td>
<td>Increase</td>
</tr>
<tr>
<td>36:1</td>
<td>0.015 0.005</td>
<td>0.010 0.005</td>
<td>0.026</td>
<td>Increase</td>
</tr>
<tr>
<td>38:6</td>
<td>0.004 0.001</td>
<td>2.93E-03 6.43E-04</td>
<td>0.009</td>
<td>Increase</td>
</tr>
<tr>
<td>38:5</td>
<td>0.004 0.001</td>
<td>2.65E-03 9.64E-04</td>
<td>0.025</td>
<td>Increase</td>
</tr>
<tr>
<td>38:3</td>
<td>0.013 0.002</td>
<td>0.010 0.003</td>
<td>0.008</td>
<td>Increase</td>
</tr>
<tr>
<td>40:3</td>
<td>0.007 0.001</td>
<td>0.005 0.001</td>
<td>0.006</td>
<td>Increase</td>
</tr>
<tr>
<td>40:2</td>
<td>0.010 0.002</td>
<td>7.05E-03 1.65E-03</td>
<td>0.012</td>
<td>Increase</td>
</tr>
<tr>
<td>42:3</td>
<td>0.011 0.002</td>
<td>8.43E-03 2.55E-03</td>
<td>0.027</td>
<td>Increase</td>
</tr>
</tbody>
</table>

¹Lipid groups. DGDG = digalactosyldiacylglycerol, MGDG = monogalactosyldiacylglycerol, PG = phosphatidylglycerol, PC = phosphatidylcholine, PE = phosphatidylethanolamine.
Table 1B: Changes in lipid profile of aphid infested leaves. The species of lipids shown in this table have shown significant changes during aphid infestation (p-value < 0.05). Also listed in this table are the averages (Avg.) of each lipid species in both infested and control leaves as percentages of the total lipid content as well as the corresponding standard deviations (STDev.) The response column shows the effect aphids have on a particular lipid species in comparison to the control.

<table>
<thead>
<tr>
<th>Species¹</th>
<th>Aphid infested</th>
<th>Non-aphid infested</th>
<th>P-value</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Avg. (%)</td>
<td>STDev.</td>
<td>Avg. (%)</td>
<td>STDev.</td>
</tr>
<tr>
<td>PI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34:3</td>
<td>1.062</td>
<td>0.122</td>
<td>0.920</td>
<td>0.146</td>
</tr>
<tr>
<td>36:6</td>
<td>0.014</td>
<td>0.003</td>
<td>0.010</td>
<td>0.005</td>
</tr>
<tr>
<td>36:5</td>
<td>0.014</td>
<td>0.003</td>
<td>0.011</td>
<td>0.002</td>
</tr>
<tr>
<td>36:4</td>
<td>9.10E-03</td>
<td>1.52E-03</td>
<td>6.80E-03</td>
<td>1.78E-03</td>
</tr>
<tr>
<td>PA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34:3</td>
<td>0.213</td>
<td>0.023</td>
<td>0.168</td>
<td>0.059</td>
</tr>
<tr>
<td>34:2</td>
<td>0.126</td>
<td>0.023</td>
<td>0.098</td>
<td>0.034</td>
</tr>
<tr>
<td>26:6</td>
<td>0.049</td>
<td>0.011</td>
<td>0.032</td>
<td>0.014</td>
</tr>
<tr>
<td>36:5</td>
<td>0.057</td>
<td>0.014</td>
<td>0.039</td>
<td>0.016</td>
</tr>
<tr>
<td>36:4</td>
<td>0.047</td>
<td>0.013</td>
<td>0.031</td>
<td>0.011</td>
</tr>
<tr>
<td>36:3</td>
<td>0.066</td>
<td>0.017</td>
<td>0.048</td>
<td>0.020</td>
</tr>
<tr>
<td>36:2</td>
<td>0.032</td>
<td>0.009</td>
<td>0.023</td>
<td>0.009</td>
</tr>
<tr>
<td>lysoPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3</td>
<td>0.012</td>
<td>0.002</td>
<td>8.20E-03</td>
<td>3.01E-03</td>
</tr>
</tbody>
</table>

¹Lipid groups. PI = phosphatidylinositol, PS = phosphatidylserine, PA = phosphatidic acid, lysoPE = lysophosphatidylethanolamine, lysoPG = lysophosphatidylglycerol.
Table 2A: Soybean genes corresponding to lipid metabolism differentially regulated by 7 days of soybean aphid feeding.

<table>
<thead>
<tr>
<th>Glyma2.0 Locus</th>
<th>Probe Set Name</th>
<th>P-Value</th>
<th>FDR</th>
<th>Fold Change&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyma.20g020800</td>
<td>Gma.10860.1.S1_a_at</td>
<td>0.000433</td>
<td>0.01062</td>
<td>2.00</td>
<td>cytosolic calcium-independent phospholipase A</td>
</tr>
<tr>
<td>Glyma.11g230100</td>
<td>Gma.221.1.S1_at</td>
<td>0.000064</td>
<td>0.00427</td>
<td>13.40</td>
<td>PHOSPHOLIPASE C 2</td>
</tr>
<tr>
<td>Glyma.18g027100</td>
<td>Gma.13672.1.A1_at</td>
<td>0.000303</td>
<td>0.00887</td>
<td>8.55</td>
<td>phosphatidylinositol phospholipase C</td>
</tr>
<tr>
<td>Glyma.08g211700</td>
<td>Gma.4902.1.S1_at</td>
<td>0.000071</td>
<td>0.00449</td>
<td>3.17</td>
<td>Phospholipase D alpha 1 (EC 3.1.4.4) (PLD alpha 1) (Choline phosphatase 1) (Phosphatidylycholine-hydrolyzing phospholipase D 1)</td>
</tr>
<tr>
<td>Glyma.13g364900</td>
<td>GmaAffx.80588.1.S1_at</td>
<td>0.000891</td>
<td>0.01522</td>
<td>2.23</td>
<td>phospholipase D alpha 1</td>
</tr>
<tr>
<td>Glyma.19g132900</td>
<td>GmaAffx.2613.1.S1_at</td>
<td>0.000606</td>
<td>0.01267</td>
<td>2.14</td>
<td>Phospholipase, patatin family</td>
</tr>
<tr>
<td>Glyma.02g013700</td>
<td>Gma.1583.1.S1_s_at</td>
<td>0.000005</td>
<td>0.00111</td>
<td>5.04</td>
<td>Lipase, class 3 (alpha/beta-Hydrolases superfamily)</td>
</tr>
<tr>
<td></td>
<td>GmaAffx.53860.1.S1_at</td>
<td>0.000006</td>
<td>0.00114</td>
<td>3.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gma.1583.1.S1_at</td>
<td>0.000025</td>
<td>0.00253</td>
<td>3.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GmaAffx.15333.1.S1_at</td>
<td>0.000001</td>
<td>0.00035</td>
<td>3.54</td>
<td></td>
</tr>
<tr>
<td>Glyma.03g159000</td>
<td>Gma.12487.1.S1_at</td>
<td>0.001572</td>
<td>0.01976</td>
<td>3.11</td>
<td>Lipase, class 3 (alpha/beta-Hydrolases superfamily)</td>
</tr>
<tr>
<td></td>
<td>Gma.12487.2.S1_at</td>
<td>0.003461</td>
<td>0.02769</td>
<td>2.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GmaAffx.65238.1.A1_at</td>
<td>0.008200</td>
<td>0.04152</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>Glyma.01g186600</td>
<td>GmaAffx.38907.1.S1_at</td>
<td>0.001451</td>
<td>0.01897</td>
<td>2.29</td>
<td>Lipase, class 3 (alpha/beta-Hydrolases superfamily)</td>
</tr>
<tr>
<td>Glyma.15g097100</td>
<td>GmaAffx.93244.1.A1_s_at</td>
<td>0.000213</td>
<td>0.00757</td>
<td>2.21</td>
<td>Lipase, class 3 (alpha/beta-Hydrolases superfamily)</td>
</tr>
<tr>
<td>Glyma.01g205900</td>
<td>Gma.4537.1.S1_at</td>
<td>0.007971</td>
<td>0.04096</td>
<td>2.00</td>
<td>Lipase, class 3 (alpha/beta-Hydrolases superfamily)</td>
</tr>
<tr>
<td>Glyma.07g089400</td>
<td>Gma.8398.1.S1_at</td>
<td>0.002038</td>
<td>0.02204</td>
<td>1.96</td>
<td>Lipase, alpha/beta-Hydrolases superfamily</td>
</tr>
</tbody>
</table>

<sup>1</sup> Genes with increased expression in response to aphids are shown in yellow cells; genes repressed by aphids are shown in blue cells.
Table 2B: Soybean genes corresponding to lipid metabolism differentially regulated by 7 days of soybean aphid feeding.

<table>
<thead>
<tr>
<th>Glyma2.0 Locus</th>
<th>Probe Set Name</th>
<th>P-Value</th>
<th>FDR</th>
<th>Fold Change</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyma.08g318400</td>
<td>GmaAffx.67834.1.S1_at</td>
<td>0.008164</td>
<td>0.04144</td>
<td>1.95</td>
<td>GDSL-motif lipase/hydrolase family protein</td>
</tr>
<tr>
<td>Glyma.14g050900</td>
<td>Gma.10574.1.S1_at</td>
<td>0.001039</td>
<td>0.01637</td>
<td>-2.46</td>
<td>GDSL-motif lipase/hydrolase family protein</td>
</tr>
<tr>
<td>Glyma.13g105300</td>
<td>Gma.7211.1.S1_at</td>
<td>0.000373</td>
<td>0.00981</td>
<td>2.18</td>
<td>alpha/beta-Hydrolases superfamily protein, BAAT/Acyl-CoA thioester hydrolase C-terminal</td>
</tr>
<tr>
<td>Glyma.03g056700</td>
<td>Gma.11043.1.S1_at</td>
<td>0.017826</td>
<td>0.06162</td>
<td>-2.02</td>
<td>Chloroplast omega-3 desaturase FAD7/8</td>
</tr>
<tr>
<td>Glyma.08g015600</td>
<td>Gma.11025.1.S1_at</td>
<td>0.007803</td>
<td>0.04052</td>
<td>-1.99</td>
<td>Cytochrome P450 monoxygenase CYP86A</td>
</tr>
<tr>
<td>Glyma.07g146800</td>
<td>Gma.13420.1.S1_at</td>
<td>0.000069</td>
<td>0.00445</td>
<td>-4.81</td>
<td>glycerol-3-phosphate acyltransferase</td>
</tr>
<tr>
<td>Glyma.12g075100</td>
<td>Gma.13818.1.S1_at</td>
<td>0.004990</td>
<td>0.03251</td>
<td>-2.73</td>
<td>3-ketoacyl-CoA synthase 12</td>
</tr>
<tr>
<td>Glyma.02g123700</td>
<td>Gma.3181.1.S1_at</td>
<td>0.000094</td>
<td>0.00520</td>
<td>2.00</td>
<td>Phosphatidylinositol 3-and 4-kinase</td>
</tr>
<tr>
<td>Glyma.13g114600</td>
<td>Gma.8773.1.S1_at</td>
<td>0.000084</td>
<td>0.00488</td>
<td>2.44</td>
<td>phosphoinositide phosphatase family protein</td>
</tr>
<tr>
<td>Glyma.17g045400</td>
<td>GmaAffx.49072.1.S1_at</td>
<td>0.001446</td>
<td>0.01895</td>
<td>2.31</td>
<td>phosphoinositide phosphatase family protein</td>
</tr>
<tr>
<td>Glyma.11g218500</td>
<td>GmaAffx.87704.2.S1_at</td>
<td>0.001362</td>
<td>0.01861</td>
<td>2.46</td>
<td>inositol 1,3,4-trisphosphate 5/6-kinase 4</td>
</tr>
<tr>
<td>Glyma.12g177300</td>
<td>Gma.8221.1.A1_at</td>
<td>0.001187</td>
<td>0.01750</td>
<td>1.99</td>
<td>phosphatidate cytidylytransferase family protein (CDP-diacylglycerol synthase)</td>
</tr>
<tr>
<td>Glyma.18g018600</td>
<td>Gma.15564.1.S1_at</td>
<td>0.000537</td>
<td>0.01182</td>
<td>2.51</td>
<td>Myo-inositol-1-phosphate synthase</td>
</tr>
<tr>
<td>Glyma.07g080800</td>
<td>Gma.7523.1.S1_at</td>
<td>0.001218</td>
<td>0.01763</td>
<td>2.18</td>
<td>Acyltransferase</td>
</tr>
<tr>
<td>Glyma.19g099400</td>
<td>GmaAffx.27818.1.A1_at</td>
<td>0.014022</td>
<td>0.05461</td>
<td>2.42</td>
<td>Esterase/lipase/thioesterase family protein</td>
</tr>
<tr>
<td>Glyma.19g099400</td>
<td>GmaAffx.27818.2.S1_at</td>
<td>0.034717</td>
<td>0.08709</td>
<td>2.24</td>
<td>Esterase/lipase/thioesterase family protein</td>
</tr>
</tbody>
</table>

1 Genes with increased expression in response to aphids are shown in yellow cells; genes repressed by aphids are shown in blue cells.
**Table 3.** Soybean genes corresponding to the oxylipin pathway differentially regulated by 7 days of soybean aphid feeding.

<table>
<thead>
<tr>
<th>Glyma2.0 Locus</th>
<th>Probe Set Name</th>
<th>P-Value</th>
<th>FDR</th>
<th>Fold Change$^1$</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyma.14g223600</td>
<td>GmaAffx.53501.1.S1_at</td>
<td>0.00001</td>
<td>0.00168</td>
<td>2.98</td>
<td>12-oxophytodienoate reductase 1</td>
</tr>
<tr>
<td>Glyma.05g180100</td>
<td>GmaAffx.23763.1.S1_at</td>
<td>0.00000</td>
<td>0.00071</td>
<td>3.37</td>
<td>Acyl-coenzyme A oxidase 2</td>
</tr>
<tr>
<td></td>
<td>GmaAffx.90466.1.S1_at</td>
<td>0.00003</td>
<td>0.00304</td>
<td>2.59</td>
<td></td>
</tr>
<tr>
<td>Glyma.12g079600</td>
<td>Gma.17493.1.S1_at</td>
<td>0.00021</td>
<td>0.00755</td>
<td>2.39</td>
<td>peroxisomal acyl-activating enzyme</td>
</tr>
<tr>
<td>Glyma.11g130300</td>
<td>Gma.702.1.S1_at</td>
<td>0.00029</td>
<td>0.00876</td>
<td>2.99</td>
<td>lipoxygenase 2</td>
</tr>
<tr>
<td></td>
<td>GmaAffx.70690.1.S1_at</td>
<td>0.00055</td>
<td>0.01194</td>
<td>2.84</td>
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</tr>
<tr>
<td>Glyma.13g030300</td>
<td>Gma.2382.1.S1_x_at</td>
<td>0.01090</td>
<td>0.04797</td>
<td>2.19</td>
<td>lipoxygenase 2</td>
</tr>
<tr>
<td></td>
<td>GmaAffx.91308.1.S1_at</td>
<td>0.00238</td>
<td>0.02355</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gma.2382.1.S1_at</td>
<td>0.00385</td>
<td>0.02889</td>
<td>2.26</td>
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</tr>
<tr>
<td></td>
<td>Gma.2382.1.S1_s_at</td>
<td>0.00280</td>
<td>0.02522</td>
<td>2.83</td>
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</tr>
<tr>
<td>Glyma.08g189600</td>
<td>Gma.1.1.S1_at</td>
<td>0.00178</td>
<td>0.02081</td>
<td>10.68</td>
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</tr>
<tr>
<td></td>
<td>Gma.1.1.A1_at</td>
<td>0.00738</td>
<td>0.03954</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>Glyma.15g026400</td>
<td>Gma.10969.3.S1_x_at</td>
<td>0.00136</td>
<td>0.01861</td>
<td>6.38</td>
<td>lipoxygenase 1</td>
</tr>
<tr>
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<td>0.00157</td>
<td>0.01979</td>
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<tr>
<td>Glyma.13g347800</td>
<td>Gma.10969.1.S1_x_at</td>
<td>0.00129</td>
<td>0.01815</td>
<td>4.05</td>
<td>lipoxygenase 1</td>
</tr>
<tr>
<td></td>
<td>Gma.10969.1.S1_at</td>
<td>0.00184</td>
<td>0.02102</td>
<td>3.70</td>
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</tr>
<tr>
<td>Glyma.07g034800</td>
<td>Gma.11166.1.S1_s_at</td>
<td>0.00110</td>
<td>0.01682</td>
<td>2.86</td>
<td>lipoxygenase 1</td>
</tr>
<tr>
<td></td>
<td>Gma.11166.1.S1_x_at</td>
<td>0.00026</td>
<td>0.00844</td>
<td>2.84</td>
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</tr>
<tr>
<td></td>
<td>GmaAffx.93591.1.S1_s_at</td>
<td>0.00302</td>
<td>0.02624</td>
<td>2.51</td>
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</tr>
<tr>
<td></td>
<td>GmaAffx.81415.1.S1_at</td>
<td>0.00548</td>
<td>0.03405</td>
<td>2.31</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gma.11166.1.S1_at</td>
<td>0.00063</td>
<td>0.01289</td>
<td>3.91</td>
<td></td>
</tr>
<tr>
<td>Glyma.11g239400</td>
<td>Gma.5756.1.S1_at</td>
<td>0.00796</td>
<td>0.04096</td>
<td>2.02</td>
<td>Lipase/lipoxygenase, PLAT/LH2 family protein</td>
</tr>
</tbody>
</table>

$^1$ Genes with increased expression in response to aphids are shown in yellow cells; genes repressed by aphids are shown in blue cells.
Table 4. Soybean genes corresponding to lipid-binding proteins differentially regulated by 7 days of soybean aphid feeding.

<table>
<thead>
<tr>
<th>Glyma2.0 Locus</th>
<th>Probe Set Name</th>
<th>P-Value</th>
<th>FDR</th>
<th>Fold Change&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyma.13g088700</td>
<td>GmaAffx.1301.22.S1_s_at</td>
<td>0.000098</td>
<td>0.00529</td>
<td>10.84</td>
<td>Annexin</td>
</tr>
<tr>
<td></td>
<td>GmaAffx.1301.68.S1_s_at</td>
<td>0.000403</td>
<td>0.01022</td>
<td>10.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GmaAffx.1301.59.S1_at</td>
<td>0.000123</td>
<td>0.00598</td>
<td>12.27</td>
<td></td>
</tr>
<tr>
<td>Glyma.13g261200</td>
<td>GmaAffx.38045.1.S1_at</td>
<td>0.000125</td>
<td>0.00598</td>
<td>5.43</td>
<td>Calcium-dependent lipid-binding (CaLB domain) family protein</td>
</tr>
<tr>
<td>Glyma.11g107300</td>
<td>GmaAffx.52789.1.S1_at</td>
<td>0.000094</td>
<td>0.00519</td>
<td>10.18</td>
<td></td>
</tr>
<tr>
<td>Glyma.17g122000</td>
<td>GmaAffx.8316.1.S1_at</td>
<td>0.001440</td>
<td>0.01890</td>
<td>2.71</td>
<td>Oleosin</td>
</tr>
<tr>
<td>Glyma.14g115500</td>
<td>Gma.3291.1.S1_at</td>
<td>0.000013</td>
<td>0.00178</td>
<td>-8.02</td>
<td>Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein</td>
</tr>
<tr>
<td>Glyma.11g251100</td>
<td>Gma.15583.1.S1_a_at</td>
<td>0.000348</td>
<td>0.02741</td>
<td>2.29</td>
<td>glycosylphosphatidylinositol-anchored lipid protein transfer 1</td>
</tr>
<tr>
<td>Glyma.14g017900</td>
<td>GmaAffx.90699.1.A1_at</td>
<td>0.000138</td>
<td>0.00635</td>
<td>2.32</td>
<td>lipid-binding serum glycoprotein family protein</td>
</tr>
<tr>
<td>Glyma.15g112500</td>
<td>Gma.4315.1.S1_at</td>
<td>0.001299</td>
<td>0.01817</td>
<td>2.26</td>
<td>Fibrillin</td>
</tr>
<tr>
<td>Glyma.08g331800</td>
<td>Gma.1421.2.S1_at</td>
<td>0.000645</td>
<td>0.01297</td>
<td>2.16</td>
<td>Sec14p-like phosphatidylinositol transfer family protein</td>
</tr>
<tr>
<td>Glyma.05g200000</td>
<td>Gma.103.1.S1_at</td>
<td>0.012266</td>
<td>0.05081</td>
<td>-2.15</td>
<td>Sec14p-like phosphatidylinositol transfer family protein</td>
</tr>
</tbody>
</table>

<sup>1</sup> Genes with increased expression in response to aphids are shown in yellow cells; genes repressed by aphids are shown in blue cells.
CHAPTER FOUR

GENERAL CONCLUSIONS AND DISCUSSION

With their entry into the Midwestern United States during the year 2000 (Hartman et al., 2001), soybean aphids have proven to be a major threat to soybean. These phloem feeding insects threaten with annual soybean yield reductions of up to 50% (Ragsdale et al., 2007) by changing soybean biochemistry to avert plant defenses. Although the mechanism by which soybean aphids bypass host defenses is still not well known, increasing efforts have been made to better characterize the relationship between soybean and soybean aphids. To date, we have evidence that both the jasmonic (JA) and abscisic (ABA) pathway play roles in soybean interactions with soybean aphids (Li et al., 2008; Studham and MacIntosh, 2013; Kanobe, 2012; Hohenstein and MacIntosh, unpublished). One of the main objectives of this research is to better understand if soybean aphids have any effect on systemic plant defenses. By examining the fatty acids palmitic (16:0), linoleic (18:2), and linolenic (18:3) acids, we hoped to determine changes in the JA-related responses both locally and systemically. While it has been very clear that soybean aphids modify local 16:0 and 18:3 content, their effect on systemic fatty acid content was unknown until this study. We have shown that systemic content of 16:0 and 18:3 are left unchanged. In addition to this, we examined the local and systemic expression levels of an omega-6 fatty acid desaturase, FAD6, and found that this transcript is downregulated only locally. Furthermore, given that past experiments (Studham and MacIntosh, 2013; Hohenstein and MacIntosh, unpublished) showed an antagonistic relationship between ABA and JA, we examined SCOF-1, an ABA induced transcription factor, and found its expression to be upregulated both locally and systemically. With these results, we propose that soybean aphids block local JA-related defenses by changing fatty acid content and
prevent the mounting of systemic defenses by activating the ABA pathway, a decoy pathway that appears to suppress JA-related defenses.

In addition to the analysis of fatty acid and gene expression at the local tissue, we also obtained lipidomics and transcriptomics data on local tissue of aphid infested plants. We found the levels of many membrane lipids to be changed, with the majority of these levels increased by soybean aphids. Of particular interest are 3 classes of membrane lipids; phosphatidic acid (PA), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). There is evidence that each of these classes of lipids plays a role in wounding or wounding-related responses in plants (Mosblech et al., 2008; Zhang et al., 2004; Blancaflor et al., 2013). PA not only serves as a source of linolenic acid, the main substrate in the JA producing oxylipin pathway, but has also been seen to be affected by ABA. Our results show increased levels of PA as well as phospholipase D, the enzyme catalyzing the formation of PA from other membrane lipids, suggesting that the induction of ABA at the local tissue also increases production of PA. However, given that PA is also produced in response to JA, the role of this phospholipid in soybean-soybean aphid interaction cannot be fully understood without further experiments. PEs are a class of membrane lipids also associated with ABA signaling. Recent studies on N-Acylethanolamines (NAEs), derivatives of PEs, have shown synergistic relationships between ABA and NAEs (Blancaflor et al., 2013). The increase in levels of PA and PE suggest the increase in induction of a local ABA signal, which is consistent with our studies on the ABA marker SCOF-1. However, even with the increase in a local ABA signal, there is significant upregulation of many oxylipin biosynthetic genes in our transcriptomic analysis. Following our hypothesis on the antagonism that ABA has to JA, therefore, we can propose that the upregulation of these genes is used for a different branch of the oxylipin pathway that does not produce JA. However, there still lies the possibility that the upregulation of these oxylipin genes
serves to overproduce JA to overcome any inhibitory effects, even if this overproduction is ultimately ineffective.

PIs have been shown to be closely related with JA responses (Mosblech et al., 2008; Mosblech et al., 2011). The increase in levels of PI correlates with our observed increase in levels of phospholipase C (PLC), the enzyme responsible for producing inositol phosphates from PIs. Inositol phosphates have been shown to accumulate during wounding, and in mutants deficient in JA biosynthesis, there is no accumulation. These results suggest that PIs and their derivatives are essential in JA-related wounding responses. However, we expect the levels of JA-related signals to be repressed given the apparent reduction in precursor 18:3 as well as the activation of the antagonistic ABA pathway. Therefore, the increase in PIs in our study may be primarily to mount a physical line of defense known as phloem sieve element occlusion. This mechanism of defense begins with production of an inositol phosphate and then a transient release of calcium from the endoplasmic reticulum to the cytoplasm of phloem sieve element cells. This assumption is consistent in our findings with increase in expression levels of several calcium-regulated lipid-binding genes.

Even with the findings in this research, the exact mechanisms by which soybean aphids are able to thrive on soybean remains unknown. More research must be done to understand the effects soybean aphids have on soybean defense at both the local and systemic levels. Our lipidomics analysis is only preliminary; therefore, deeper analysis must be done to characterize the changes in lipids. Furthermore, this analysis was done only at the local tissue. Given the nature of the systemic repression of JA by ABA proposed in this research, it would be beneficial to perform a lipidomics analysis on systemic tissue.
REFERENCES


Kanobe, C. (2012). Fatty acid changes in soybean (Glycine max) under soybean aphid (Aphis glycines) infestation and their implications on plant defense against insects. Graduate Theses and Dissertations, 1065-1072.


ACKNOWLEDGEMENT

First and foremost, I would like to thank my major professor and mentor, Dr. Gustavo MacIntosh, for not only guiding me though my research over the past few years, but also for allowing me, a undergraduate sophomore at the time, to begin working and undertaking independent projects in his lab. He was also willing to take me under his wing as a B.S./M.S. student and worked hard in ensuring I had all I needed to complete my degree. Thank you for all the opportunities and guidance you have given me!

I would also like to thank my parents, Ha Hoang and Tuong Nguyen, in supporting me in everything I do and teaching me perseverance and patience for when it mattered most. I especially want to thank my older brother, Khoa Nguyen, for not only advising and supporting me, but also for pushing me to my limits to ensure that I succeeded. I owe the path that took me here today to you and mom and dad.

Last but not least, I would like to thank all the members of the MacIntosh lab, past and present, who have taught me, helped me, and laughed with me throughout all of these years. Special thank you to Jessica Hohenstein, Stephanie Morriss, Charles Kanobe, Martha Ibore, Ayesha Riaz, and Ting Lau!