PATHOGEN TRIGGERED PLANT VOLATILES INDUCE SYSTEMIC SUSCEPTIBILITY IN NEIGHBORING PLANTS

A Dissertation by

NASIE NOEL CONSTANTINO

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Chair of Committee,	Michael Kolomiets
Committee Members,	Charles Kenerley
	Libo Shan
	Scott Finlayson
Head of Department,	Leland Pierson III

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ABSTRACT

This project elucidates the effect of volatile organic compounds (VOCs) emitted by maize infected with fungal pathogens on disease progression in neighboring plants. To protect themselves from herbivory by insects, plants initiate a multifaceted defense response. In contrast to direct defense, characterized by the production of toxic metabolites, indirect defense relies on volatile emissions to attract predators of the insect herbivores as well as warning neighboring plants to prepare for insect attack. This biochemically diverse bouquet of volatiles produced in response to insect feeding is known as herbivore-induced plant volatiles (HIPVs). One subgroup of HIPVs is Green Leaf Volatiles (GLVs), which govern plant-plant and plant-insect communication, as well as endogenous defensive signaling. Despite the strategic role of GLVs in insect defense, their function during microbial interactions has been widely overlooked. Herbivory induced GLVs prime neighboring plants against impending insect attack by enabling them to produce greater levels of jasmonic acid (JA) when challenged with an infestation. This priming phenomenon is called induced systemic resistance (ISR). Despite the strategic role of GLVs in insect defense, their function in direct and indirect defenses against pathogens has been largely overlooked. Unexpectedly, pathogen-induced plant volatiles (PIPVs) induce systemic susceptibility (ISS) in neighboring plants, rather than ISR, as the receiver plants exposed to PIPVs from infected plants became significantly more susceptible to *C. graminicola* and *Cochliobolus heterostrophus*. Susceptibility in neighboring plants is caused by the release of GLVs from infected plants that induces the positive regulation and biosynthesis of JA in neighboring plants. JA has been shown to promote susceptibility to *C. graminicola*. *LOX2, LOX5, LOX12* and *OPR2* are PIPV and GLV inducible genes that are involved in JA regulation and biosynthesis in maize and are

vital to the induction of ISS in PIPV exposed receiver plants. In summary, this research illustrates a novel model for explaining how pathogen infections may spread under epidemic-prone conditions in the field. In this model, volatile emissions from infected plants predispose neighboring plants to become a more suitable host for an imminent infection.

DEDICATION

To my service dog Axel, my mother Lisa Constantino, my father Steve Constantino, my great aunts Edith and Joanne Constantino, and my grandmother Betty Woodman.

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All work for the dissertation was completed by the student, with the help from several undergraduate students, Hannah Price, Spring Laferriere, Colette Khouri, and Lorenzo Washington from the Department of Bioenvironmental Sciences. Assisted by Zack Gorman from the Department of Plant Pathology and Microbiology to use and analyze volatile organic compounds using a GC-MS. GC-MS was provided my Scott Finlayson. All phytohormone and fungal biomass analysis was performed by Dr. Eli Borrego of the Plant Pathology and Microbiology Department.

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NOMENCLATURE

LOX	Lipoxygenase
OPR	Oxo-phytodienoate reductase
HIPVs	Herbivore-induced plant volatiles
PIPVs	Pathogen-induced plant volatiles
VOCs	Volatile Organic Compounds
HPL	Hydroperoxide lyases
GLVs	Green Leaf Volatiles
ISR	Induce Systemic Resistance
ISS	Induce Systemic Susceptibility
JA	Jasmonic acid
MeJA	Methyl jasmonate
JA-ILe	Jasmonoyl-isoleucine
13-HPOTE	13-hydroperoxy octadecatrienoic acid
AOS	Allene oxide synthase
AOC	Allene oxide cyclase
12-OPDA	(+)-12-oxo-phytodienoic acid
FAW	Fall armyworms
SA	Salicylic acid

SAR	Systemic Acquired Resistance
PR	Pathogenesis-related
PDA	Potato dextrose agar
rpm	Revolutions per minute
PET	Polyethylene terephthalate
DEPC	Diethyl pyrocarbonate
HSP	Heat shock proteins
HSF	Heat shock factors
Nramp1	Natural resistance-associated macrophage protein
GPCR	G protein coupled receptors
ORs	Olfactory receptors

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INTRODUCTION

Volatiles Induced by Herbivory and Pathogen Infection

Upon herbivore infestation, plants emit a complex blend of herbivore induced plant volatiles (HIPVs) including green-leaf volatiles (GLVs), which are emitted immediately upon tissue damage. GLVs and other volatiles produced during herbivory belong to a more general group of volatiles known as volatile organic compounds (VOCs). GLVs emitted by damaged plants are perceived as caterpillar location cues for herbivore predators, such as parasitic wasps, and serve as molecular signals to aid in the defense of the infested plant (Furstenberg et al., 2013; Christensen et al. 2013). GLVs are also emitted by mechanical wounding, however the specific blends of GLVs differ from those produced in response to insect feeding. During caterpillar herbivory, caterpillars regurgitate several classes of insect-derived elicitors in their oral secretions, including the most well studied insect elicitor called volicitin. Volicitin was shown to induce the major insect defense hormone, jasmonic acid (JA), and HIPVs synthesis that result in heightened defense responses (Turlings et al., 2000). Several GLVs emitted by plants consumed by caterpillars include (Z)-3-hexenal, (Z)-3-hexenol, (Z)-3-hexenyl acetate, and (E)-2-hexenal (Christensen et al., 2013; Farag et al., 2005). Of these, (Z)-3-hexenal, (Z)3-hexenol, (Z)-3-hexenyl acetate have been shown to induce jasmonic acid (JA) in neighboring plants to prepare those plants for an impending insect attack, a phenomenon generally referred to as induced systemic resistance (ISR) (Ton et al., 2007). In maize, the model host for this research, LOX10 is the sole lipoxygenase isoform responsible for the synthesis of the GLV oxylipin in tissues containing chlorophyll (Christensen et al., 2013).

GLV release in response to insect herbivory has been well-studied, but minimal information has been reported on GLVs produced in response to pathogen infection. (Z)-

3hexenal, (E)-2-hexenal, (Z)-3-hexenyl acetate were found in cereal crops challenged with either beetles or *Fusarium* spp. infection (Piesik et al., 2010). Both beetle herbivory and infection also induced the production of the volatiles β-linalool and β-caryophyllene (Piesik et al., 2010). β-linalool is a terpene alcohol that has been implicated in the defense against insects by increasing egg predation rates by predators (Kessler et al., 2001). In maize, β-caryophyllene is a sesquiterpene that has been implicated in indirect defense in maize via attraction of parasitoids. β-caryophyllene is produced by the maize terpene synthase 23 (TPS23) gene (Kollner et al., 2008). Interestingly, besides being GLV-deficient, maize *lox10* mutants emit substantially lower levels of terpene volatiles in response to herbivory (Christensen et al., 2013).

In maize, upon infection with a mixture of four *Fusarium* species, plants emit increased levels of GLVs ((Z)-3-hexenal, (E)-2-hexenal, (Z)-3-hexenol, (E)-2-hexenol, (Z)-3-hexenyl acetate and 1-hexyl acetate), terpenes (pinene, myrcene, Z-ocimene, linalool, caryophyllene), and shikimic acid pathway derivatives (benzyl acetate, methyl salicylate, and indole) (Piesik et al., 2011). Exposure of maize seedlings to these volatiles resulted in increased VOC production in a distance-dependent manner. Uninfected plants placed 1 m away from infected plants produced a higher concentration of VOCs than those placed 3 m away (Farmer, 2001). It should also be noted that infection of maize leaves induces a greater VOC production compared to a root infection (Piesik et al., 2011). My research focuses solely on elucidating the role of VOCs, specifically GLVs, produced by infected maize leaves and their effects on neighboring plants.

Green Leaf Volatiles (GLV) Biosynthesis

Identifying the role for GLVs in plant to plant signaling in response to Colletotrichum graminicola infection is the primary focus of my research. GLVs are synthesized by the lipoxygenase (LOX) pathway. In this pathway, fatty acids are oxygenated at 9- or 13-carbon position of linolenic or linoleic acids to produce hydroperoxy fatty acids, which are then shunted into at least seven distinct sub-branches to produce a diverse group of oxygenated the fatty acid metabolites known as oxylipins (Figure 1).

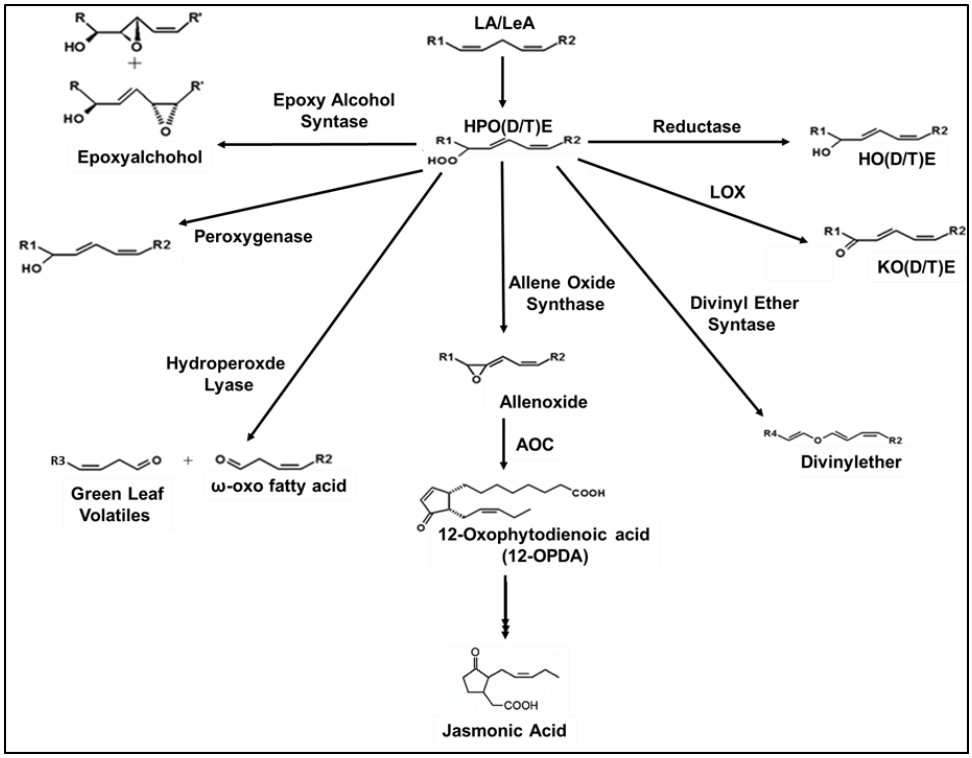

Figure 1: The lipoxygenase pathway showing the seven distinct branches of the LOX pathway that are responsible for oxylipin synthesis in plants. The principal branches of the LOX pathway in this dissertation are the JA-Producing AOS pathway and the GLV-producing HPL pathway.

GLVs are one small group of an estimated plant 650 oxylipins that have been identified thus far (Borrego and Kolomiets, 2016). In plants, little is known about physiological roles for the

majority of oxylipins. However, GLVs play an important role as molecular signals in direct and indirect defense responses against insect herbivory in maize (Christensen et al., 2013). In addition, several specific GLV molecular species have been demonstrated to have direct potent antimicrobial activities when applied exogenously *in vitro* to fungal and bacterial pathogens (Prost et al., 2005).

In the maize GLV pathway, a single 13-LOX isoform, LOX10 (located in the chloroplast), is responsible for GLV biosynthesis in leaves by adding molecular oxygen at position 13 of linolenic acid substrate to produce 13-hydroperoxide of linolenic acid, 13hydroperoxy octadecatrienoic acid (13-HPOTE) (Figure 2).

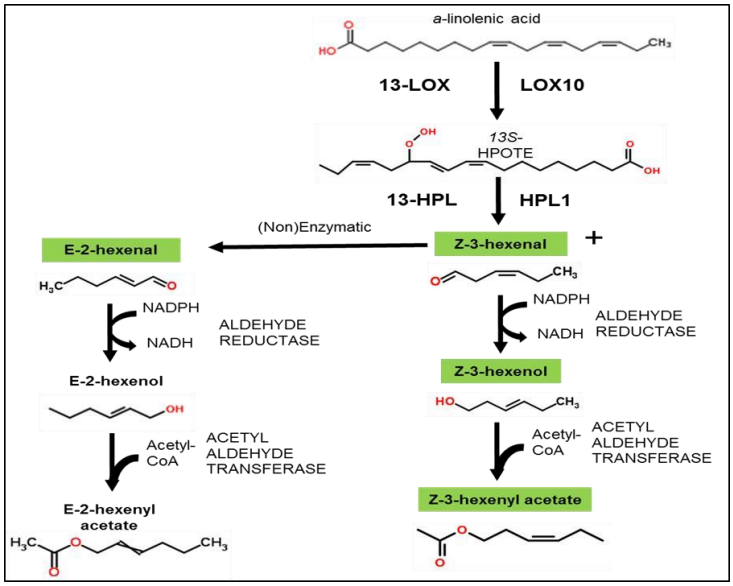

Figure 2: Biochemical pathway for the biosynthesis of GLVs. LOX10 is the 13-LOX responsible for oxygenating linolenic acid at the carbon 13 position to create hydrogen peroxides of fatty acids which in turn are cleaved by HPL1, a 13-hydroperoxide lyase, at the C12-C13 bond to produce (Z)-3-Hexenal. (Z)-3-Hexenal is reduced to form (Z)-3-Hexenol and a portion of the (Z)-3-Hexenol is converted into the more stable and less reactive (Z)-3-hexenyl acetate. (Z)-3-Hexenal, (Z)-3-Hexenol, and (Z)-3-hexenyl acetate are the key GLVs studied in this research.

This primary product of the LOX10 reaction is cleaved by the 13-hydroperoxide lyase, HPL1, at the C12–C13 bond to produce (Z)-3-hexenal. (Z)-3-hexenal is reduced to form (Z)-3hexenol that is further converted into a more stable and less reactive (Z)-3-hexenyl acetate.

(Z)-3-hexenal can also be converted spontaneously or enzymatically into (E)-2-hexenal. Additionally, (Z)-3-hexenal is spontaneously oxygenated to form 4-hydroperoxy-(E)-2hexenal, 4-hydroxy-(E)-2-hexenal or 4-oxo-(E)-2-hexenal (Matsui et al., 2012). A modified diagram of the pathway that includes known maize GLV biosynthesis genes, *LOX10* and *HPL1*, is shown in Figure 2. In total plants produce a total of 35 GLVs, but for this dissertation (Z)-3-hexenal, (Z)-3-hexenol, (Z)-3-hexenyl acetate, and (E)-2-hexenal will be studied (Ruther, 2000).

Maize Lipoxygenases

LOX10, a 13-LOX, is not only vital for GLV biosynthesis, but is also responsible for engaging several GLV-induced 9-LOX genes for the positive regulation of JA via enigmatic 9-oxylipin signaling. The LOX pathway is initiated with the cleavage of polyunsaturated fatty acids, linoleic (18:2) and α-linolenic (18:3) acids, from cell membranes by diverse lipases. These fatty acids are deoxygenated by either 9- or 13-LOXs to form 9- and 13-hydroperoxides, respectively. 9-LOXs are comprised of a subfamily of proteins which share a relatively high amino acid sequence identity (>60%), while the 13-LOXs are comprised of genes that have a moderate sequence identity (~35%) (Vernooy-Gerritsen et al. 1984; Park et al., 2010). The 9- and 13- hydroperoxides act as substrates for seven downstream branches of the LOX pathway. The seven pathways include peroxygenases, divinyl ether synthases, reductases, epoxy alcohol synthases, allene oxide synthases (AOSs) and hydroperoxide lyases (HPLs) (Figure. 1).

The maize B73 genome contains 13 LOX genes (Borrego and Kolomiets, 2016). They are clustered into 2 distinct groups the 9-LOXs and the 13-LOXs (Figure 3). The 9-LOX group consists of LOX1 through LOX5 and LOX12 (Park et al., 2010; Christensen et al., 2014), while the 13-LOX group consists of LOX7, LOX8, LOX9, LOX10, LOX11 and LOX13 (Christensen et al., 2013).

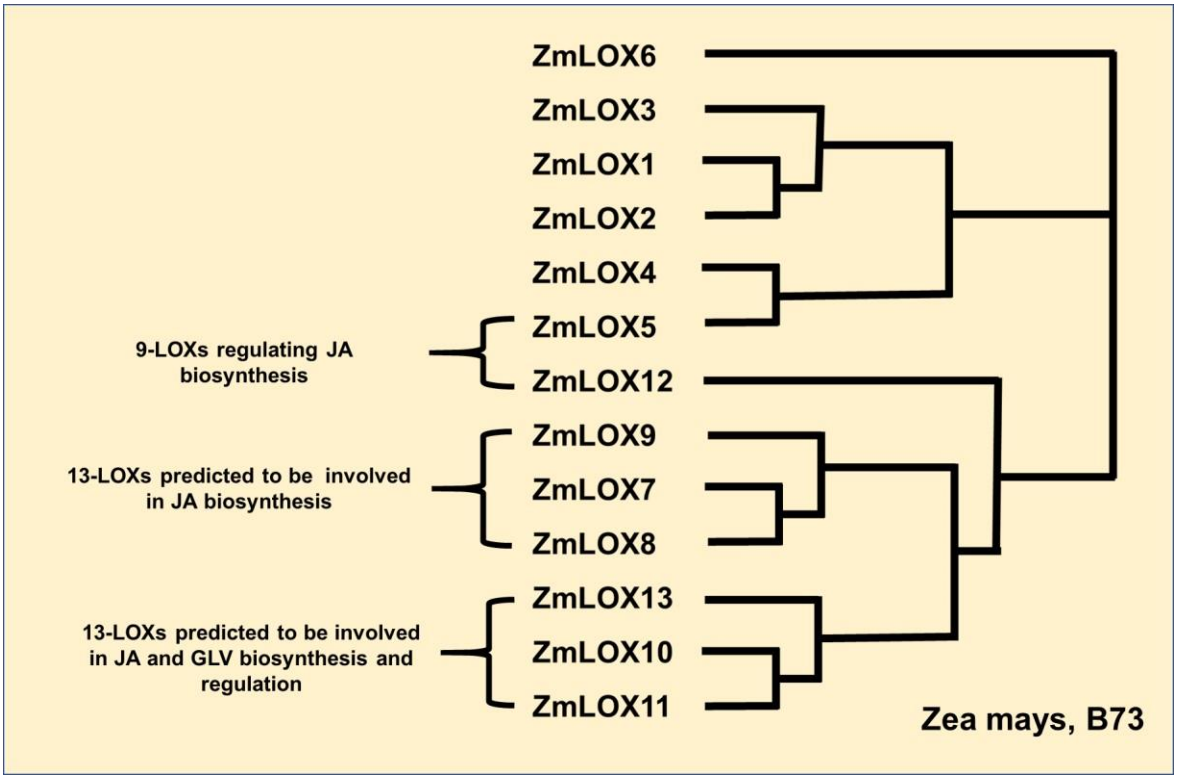

Figure 3: Phylogenetic relationship between the characterized 9- and 13- lipoxygenases. B73 maize inbred line has thirteen members that are divided into two main groups. The first group is the 9-LOXs (LOX1, 2, 3, 4, 5, 12) and the second is 13-LOXs (LOX7, 8, 9, 10, 11, 13) (Gao et al. 2008). 13-LOXs are known to be involved in JA and GLV production. 9-LOXs are less understood, but LOX5 and LOX12 have been shown to be involved in the regulation of JA biosynthesis (Park et al., 2010; Christensen et al., 2014).

The 13-LOX group is mainly involved in the production of JA and GLVS. 9-LOX-mediated branches produce numerous 9-oxylipins, functions of which are not well understood in any plant species. Interestingly, 9-oxylipins produced by at least two of these genes, LOX5 and

LOX12, appear to be hormone-like signals required for normal induction of JA in response to wounding and to pathogen infection (Christensen et al., 2014; Park, 2011). To date, the functions of the majority of the maize LOX genes have not been characterized with the notable exception of LOX3, LOX8, LOX10 and LOX12 (Gao et al., 2007, 2008, 2009; Christensen et al., 2013, 2014). LOX3 is a 9-LOX gene predominantly expressed in roots and was shown to negatively regulate ISR in response to the beneficial fungal endophyte *Trichoderma virens* (Constantino et al., 2013). In maize LOX12 is a monocot-specific 9-LOX that is required for immunity against *Fusarium verticillioides* that produces an unknown 9-oxylipin signal that positively regulates JA synthesis in response to infection (Christensen et al., 2014). LOX8 and LOX10 are 13-LOXs and are the major isoforms responsible for JA and GLV synthesis, respectively (Christensen et al., 2013). The biochemical and physiological functions of the remaining LOXs in maize are under investigation in the Kolomiets laboratory by utilizing nearisogenic inbred lines disrupted in each of the genes by insertions of *Mutator* transposable elements. My research project involves LOX that either produce GLVs directly (LOX10) or are GLV-inducible and produce novel 9-oxylipins (LOX2, LOX5 and LOX12).

Jasmonic Acid Facilitates Pathogenesis by *C. graminicola*

The 13-oxylipin JA is a widely-studied lipid-derived hormone involved in numerous physiological processes, such as reproductive development, seed germination, leaf senescence, root formation, anther development, tendril coiling, and responses to biotic and abiotic stresses (Yan, Borrego and Kolomiets, 2013; Browse et al., 2009; Avanci et al., 2010). JA formation is similar to the production of GLVs in that they both begin with the formation of LOX-derived 13-HPOTE in the chloroplast. The 13-HPOTE is converted into allene oxide by allene oxide synthase (AOS). Allene oxide is subsequently transformed into cyclopentenone by allene oxide

cyclase (AOC). The resulting (+)-12-oxo-phytodienoic acid (12-OPDA) is translocated by an ABC transporter from chloroplast to peroxisome. Once in the peroxisome, 12-OPDA is reduced by 12-oxo-phytodienoate reductase, which in maize is encoded by isoforms 7 and 8 (OPR7 and OPR8), followed by three beta-oxidation steps to form JA and its derivatives (Borrego and Kolomiets, 2013). JA and its derivatives, including methyl jasmonate (MeJA) and jasmonoyl-isoleucine (JA-Ile) are referred to as jasmonates (JAs) (Farmer et al., 2003). Maize *opr7opr8* double mutants are deficient in JA (Yan et al., 2012). While JA deficiency results in complete loss of immunity against necrotrophic *Pythium* spp. and *Fusarium verticillioides* (Christensen et al., 2014), *opr7opr8* double mutants are highly resistant to ASR caused by the hemibiotrophic *C. graminicola* (He, and Yan, unpublished). These surprising results suggest that, at least in case of *C. graminicola*, JA facilitates pathogenesis and thus, may be considered as a susceptibility factor. Since JA levels increase in plants exposed to HIPV, I hypothesize that the major mechanism underling ISS in the receiver plant exposed to infected neighbor volatiles is improper activation of JA. While suitable for defense against chewing insects, JA promotes pathogenesis by hemibiotrophs reminiscent of the pathogenicity factor produced by *Pseudomonas syringae* called coronatine, a functional and structural mimic of JA-Ile (Katsir, 2008; Mittal and Davis, 1995). Testing this hypothesis is one of the objectives of my research.

JA-Ile is the biologically active form of JA and is produced by conjugation of JA to the amino acid isoleucine by the enzyme JAR1. Once formed, JA-Ile binds to the F-box protein COI1 receptor that is a component of the ubiquitin lyase E3 complex called SCFCOI. This binding causes a change in the conformation of COI1 and that eventually results in binding JAZ proteins. JAZ is a transcriptional repressor of the major transcription factor MYC2

required for the activation of expression JA-responsive genes. JAZs are ubiquitinated and targeted for proteasome mediated degradation. As soon as JAZs are degraded, MYC2 is no longer repressed and stress-induced JA-mediated defenses are activated (Wasternack and Hause, 2013).

Anthracnose Leaf Blight Pathogen of Maize

Maize is one of the most important crops produced worldwide (Wu and Guclu, 2013) and has become of the model organisms for the study of diverse physiological processes in monocots (Strable and Scanlon, 2009). Two major diseases of maize are anthracnose leaf blight (ALB) and anthracnose stalk rot (ASR), which are caused by *C. graminicola* (Bergstrom and Nicholson, 1999; Jamil and Nicholson, 1991). *C. graminicola* is responsible for over one billion dollars of yield lost in the United States annually and is a significant threat to the global food supply (Frey et al., 2011). The main causes of yield losses in maize are lodging due to ASR and low kernel weight as a result of by ALB (Dodd, 1980). Losses caused by *C. graminicola* have been predicted to increase in the coming years due to agriculture practices which utilize monocultures, along with global climate change (Wu et al., 2002; Frey et al., 2011). *C. graminicola* is a well-studied hemibiotroph capable of infecting most plant tissues and is a filamentous ascomycete. When seedlings are infected with *C. graminicola,* lesions first develop on the lower leaves and will progressively move upward as the plant develops.

ALB's primary source of inoculum is infected crop residue from previous years. ASR infection is aided by wounding or during senescence of stalk tissue. Upon infection, *C. graminicola* forms appressoria and a penetration peg for the forceful penetration of plant epidermal cells. Once these cells have been breached, the infection hyphae are formed and begin absorbing

nutrients from the symplast by invaginating host plasma membrane (Sukno et al., 2008). The pathogen is biotrophic during the first 48 h and switches to necrotrophic life style between 48 and 72 h after the initial infection. It is therefore assumed, but not experimentally demonstrated that during the biotrophic phase, SA plays a major role in defense against this pathogen. However, during necrotrophy, the pathogen engages JA-mediated pathways as a major basal defense mechanism, as is the case for resistance against necrotrophs.

Southern Corn Leaf Blight of Maize

Like *C. graminicola*, *Cochliobolus heterostrophus* is a major fungal pathogen of maize and is found throughout the world (White, 1990). *C. heterostrophus* is the cause of Southern Corn Leaf Blight, which is a historically significant corn disease. In 1970, *C. heterostrophus* race T was the cause of an infamous epidemic in the United States that resulted in the loss of most of maize yield for that year. The disease was so rampant because of the widespread planting of highly susceptible maize lines that contained the Texas male sterile cytoplasm. *C. heterostrophus* race T was able to infect and kill any Texas male sterile cytoplasm containing maize lines because they are especially sensitive to the fungal host-specific toxin called Ttoxin (Hooker at al., 1970). The disease is most prevalent in areas that have a warm and humid climate, such as southeastern US, Africa, and parts of Asia. The pathogen is spread through leaf litter and the spores can be produced within days after initial infection. The spores are transported via wind and once they have landed on a suitable host they germinate and penetrate through stomata, leaf cuticle, or the epidermis (Manching et al., 2014).

Fall Armyworms

Similar to fungal pathogens, insects are a large concern for maize production as herbivory causes reduced yields and profit loses. *Spodoptera frugiperda*, commonly known as fall armyworm (FAW), is a devastating agricultural pest predominantly found in the Western Hemisphere. FAW has broad host range, is capable of long distance flight, and has been recently reported to have developed resistance to the widely used pesticides (Nagoshi et al., 2017). The FAW life cycle takes 30 to 90 days to complete depending on the season and comprises six instar larva phases. Larvae cause damage by consuming all leaf tissue and by burrowing into ears to devour kernels, but leave leaf, veins, and stalks intact (Foster, 1989). The absolute ferocity in which a mass of larvae can defoliate an entire field is how they acquired the name armyworm (Sparks, 1979). Recently, FAW has become a pest of an epidemic proportion in Africa and is projected to cause a $3 billion loss in the next 12 months (Stokstad, 2017). The growing economic significance of this pest to world agriculture is why I selected FAW for my comparative analyses of the effect of HPIVs and PIPVs on disease progression.

Induced Systemic Resistance (ISR)

Induced resistance is defined as a state of enhanced defense capacity elicited by prior biotic or abiotic stimuli. This resistance is effective against a broad spectrum of pathogens and pests, such as bacteria, fungi, nematodes, and insects. One form of induced resistance is systemic acquired resistance (SAR) (Vallad et al., 2004). SAR can be activated by virulent, avirulent, and non-host pathogen infections of above- or below-ground tissues. SAR activation

requires the phytohormone salicylic acid (SA) and is associated with increased expression of pathogenesis-related (PR) proteins prior to infection. Unlike SAR, induced systemic resistance (ISR) relies on priming and the JA and ethylene pathways for activation (Vallad et al., 2004). Upon herbivory, plants produce HIPVs, specifically GLVs, that not only signal to herbivore predatory insects, such as wasps (Christensen et al., 2013), but also prime neighboring plants for accelerated JA-dependent defense gene expression in response to insect feeding (Stam et al., 2014; Ballare et al., 2010). Upon priming, in contrast to SAR, plant defense pathways are only activated once challenged by pathogens or insects. The primed plants respond faster, with greater intensity, and for a longer period of time than plants that have not been primed (Vallad et al., 2004). Based on this knowledge, I initially hypothesized that PIPVs could also induce ISR in neighboring plants similar to the widely-reported effects of HIPVs on subsequent insect infestation. However, contrary to my expectations, the results presented below has shown that in maize infected with *C. graminicola*, PIPVs predispose neighboring plants to greater susceptibility to imminent infection. My research examined this unexpected phenomenon that we have termed induced systemic susceptibility (ISS).

MATERIALS AND METHODS

Plant Materials

All plant material used for this research were grown on light shelves under a 16 h light and 8 h dark cycle. The average room temperature ranged between 22-26^{o}C. Plants were grown in 7 cm diameter pots in Strong-Lite® commercial soil (Universal Mix, Pine Bluff, AZ, USA) until they reached a V4 developmental stage, indicated by the appearance of a collar on the fourth leaf. LOX2 (*lox2-1* allele), LOX5 *(lox5-3* allele) (Park, 2011), LOX10 (*lox10-3* allele) (Christensen et al., 2013), LOX12 (*lox12-1* allele) (Christensen et al., 2014), OPR7 (*opr7-5* allele), and OPR8 (*opr8-2* allele) (Yan et al., 2012) were the mutants used for this research along with near-isogenic B73 maize inbred (wild type), which was used as a recurrent parent for production of the mutants. ISS was also tested in two popcorn lines (I29 and HP301). Seeds were obtained from 2012-2016 summer growing seasons in College Station, TX. All mutants were advanced to the BC7 genetic stage in B73 genetic background.

Fungal Materials

C. graminicola (teleomorph, *Glomerella graminicola)* and *C. heterostrophus* were cultured at room temperature on potato dextrose agar (PDA) for 2 weeks under fluorescent lights. After 2 weeks, 30 ml of sterile distilled water (SDW) was poured onto the surface of the PDA. The surface was then scraped with a polystyrene cell spreader to loosen the conidia from the mycelium. The suspended conidia were then filtered through cheese cloth into a 50 ml Falcon tube to separate mycelia from the conidia in the suspension. SDW was added to the 50 ml Falcon tube to raise the volume to 40 ml. The conidia were centrifuged twice at 3,000 rpm for 3 min with decanting the supernatant and re-suspending conidia in 40 ml of SDW.

Final re-suspension was made to produce 10^6 conidia/ml how determined Suspensions were used to infect plants within 2 h of spore preparation.

Plant Inoculation

Two types of inoculation techniques were used, and all inoculations were conducted at approximately 12pm to avoid any effect of the circadian clock on the infection process. Spray inoculation was performed only for emitter infections using an atomizer (converted Misto® Olive Oil Sprayer in Brushed Aluminum, Garden City, NY, USA). Forty ml of inoculum was used to infect 20 plants. Plants were placed in vertical trays and sterile water was added to the bottoms of the trays to encourage a moist environment. After inoculation, the trays were covered with Glad Press 'N' Seal® (GLAD, Oakland, California, U.S.) to prevent drying. After 24 h, the inoculated plants were removed from the trays and placed onto the light shelves. The infected plants were separated from mock treated emitters and receivers at this time to prevent volatiles from affecting other treatments. Based on previous publications (Holopainen, 2004), optimal distance between emitters and receivers is 3 m. Two days after initial infection, emitter plants were placed into the glass air flow system along with receivers.

Drop inoculation was used to test severity of disease on the receiver plants. In contrast to spray inoculation, plants were drop inoculated while laying horizontal and their 3rd leaf taped flat to prevent the inoculum from rolling off. Each plant was inoculated at 6 distinct locations with 10 µl of the inoculum in a zig-zag pattern relative to the midvein of the leaf. Water was added to the trays to provide moisture and covered with Glad Press 'N' Seal® to produce a humid environment to facilitate infection processes. After 24 h, plants were removed from the trays and placed in the light shelves until lesions visibly developed, averaging 5-7 days.

Glass Air Flow and Volatile Collection System

A glass air flow system was built specifically for this research and used for volatile collection, as well as to expose receiver plants to emitter volatiles. Emitter plants were held in a glass chamber that is connected to the receiver chamber via 3/8 in ID tubing that allows for unidirectional air flow. All chambers were 6.5-gallon glass carboys that had their bottoms removed to allow plants to be placed inside (Fine Vine Wines, part no. 5159, Carrollton, TX, USA). The air pump is used to push air through the emitter chamber into the receiver chamber (Figure 4a). Air is pumped into the emitter chamber at 3.5 lpm and filtered through a Campbell Hausfeld PA208503AV filter to remove any outside contaminates before entering the chambers. All tubing and plastic used in the system is made from polyethylene terephthalate (PET) plastic (United States Plastic Corp., Lima, Ohio, USA). PET plastic does not emit or absorb volatiles that could interfere with the experiments.

For volatile collection, plants were placed in the glass emitter chamber and air was pulled out of the system and through a Super-Q filter, were the collected volatiles are trapped, using a Zeny™ 3,5CFM Single-Stage vacuum pump (Zeny products, Guangdong, China). Air was passively scrubbed via activated charcoal (Figure 4b). The Super-Q filter consists of 75 mg of Alltech Super-Q® adsorbent material (Analytical Research Systems, Inc., Gainesville, FL, USA) packed tightly into a 6 mm × 4 mm × 7 in Supelco glass tube (Supelco, Bellefonte, PA). All volatiles were collected from 9 am to 12 pm with an average room temperature of 24^{o}C. Volatiles were eluted from the Super-Qs into glass inserts (MicroSolv Technology Corporation, LeLand, NC) using 300 µl of dichloromethane. Samples were sealed with Parafilm M (Bemis Company, INC.) and stored at -80^{o}C to prevent evaporation.

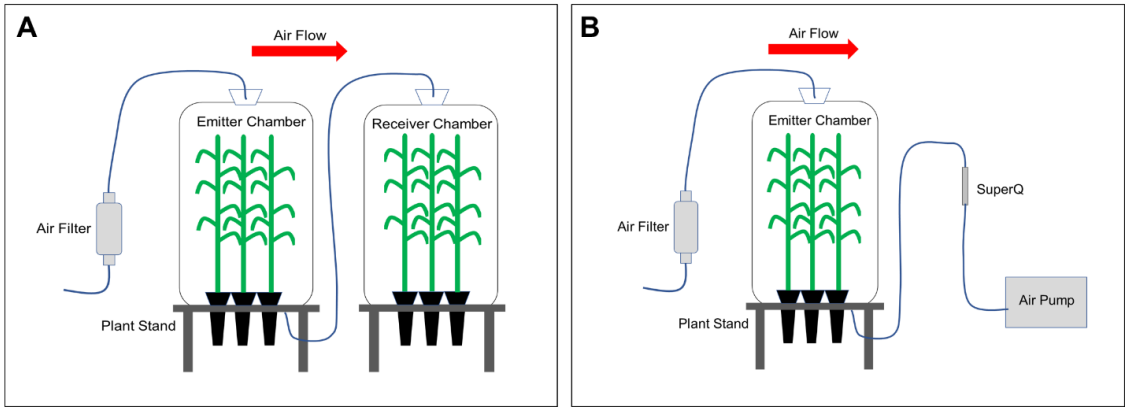

Figure 4: Schematic drawing of the glass air flow system. **(A).** Experimental set-up used to expose receiver plants to volatile organic compounds (VOCs) from the receivers. A continuous stream of charcoal-filtered air was passed through the system from the emitter chamber to the receiver chamber. **(B).** Experimental set-up used to collect VOCs from the emitter plants. Air was filtered through charcoal and suctioned out of the emitter chamber through a SuperQ for volatile collection.

Xylem Sap Collection and Application to Seedling Stems

WT maize plants were grown to the V4 developmental stage, inoculated with *C. graminicola* or sterile water (mock), and used as emitters. Twenty-four hours before receivers were exposed to the emitters, the receivers were watered until the soil was completely saturated. Forty-eight hours after the initial emitter infection, receivers were exposed to emitters for 3 h. After exposure, receivers were placed in a warm, high light intensity area and decapitated at an angle with a scalpel above the first leaf. Xylem enriched sap was collected as described in Constantino et al. (2013). Briefly, the first droplet of sap was discarded to reduce wound related molecules in the sap and subsequent droplets were collected for 3 h. All xylem sap samples were stored on ice during the collection process. The plants were periodically recut when the cut site sealed and the flow of sap diminished. Sap was frozen with liquid N2 and stored at -80°C until analysis. To reduce the viscosity, xylem sap was diluted 1:1 with sterile distilled water. Plants were then placed horizontally on a sterilized tray and two 1 cm long stem

incisions were made between the first and second leaves. The incisions were made halfway through the depth of the stem. Twenty µl of diluted sap was added to each incision.
Tape was used to seal the wound sites and ensure that the sap remained within the plant.

Gas Chromatography–Mass Spectrometry

Volatile samples were analyzed using an Agilent 7890A/5975C XL GC-MS that was equipped with a 0.25 mm x 30 M DB-5MS column (0.25m film) with pulsed split-less injection (7693A). Helium gas was used as a carrier (0.75 mL/ min). The inlet was maintained at 250^{o}C, while the oven was increased from 45^{o}C (2.25 min initial hold) to 250^{o}C for 40^{o}C per minute, held at 250^{o}C for 3 minutes, and then increased to 290^{o}C for 40^{o}C per minute. The ion source temperature was maintained at 230^{o}C and the quadrupole was heated to 150^{o}C. The ion source was operated in electron impact mode, scanning from 35 to 450 m/z with a scan time of 0.9 s and an interscan delay of 0.1 s (Ruther, 2000). Non-targeted volatile analysis was performed by using a volatile library. Target volatiles were quantified using an internal standard of 100 µM of octane and external standards ranging from 10-500 µM (Fisher Chemical, Waltham, Massachusetts, USA). External standards included (Z)-3-hexenal, (Z)-3hexenol, (Z)-3-hexenyl acetate, and (E)-2-hexenal.

Ergosterol Extraction and Analysis

Ergosterol was measured to estimate fungal biomass from infected receivers. Infected plant leaves were incubated in the dark in a solvent mixture containing a 2:1 mixture of chloroform and methanol for 24 h. The solvent was then filtered through a 0.2 µm nylonmembrane pore syringe filter and centrifuged to remove any remaining particulates. Ten µl of the extract was added to 90 µl of 20 µM C^{13}-cholesterol (cholesterol-25, 26, 27-13C;

Sigma cat. # 3707678) in methanol as an internal standard. Samples were analyzed with liquid chromatography-mass spectrometry utilizing atmospheric photochemical ionization ((+)APCI-MS/MS) (Headley et al., 2002). The column used was an Ascentis Express C-18 Column (3 cm X 2.1 mm, 2.7 um) connected to an API 3200 LC/MS/MS with multiple reaction monitoring (MRM) with an injection volume of 2 µl and the isocratic mobile phase consisting of methanol at a flow rate of 200 µl/min.

Phytohormone Extraction and Analysis

Five hundred µl of the phytohormone extraction buffer (1-propanol/water/ HCl [2:1:0.002 v/v/v]) and10 µl [5 uM solution] of deuterated internal standards: d-ABA ([2H6] (+)-cis,transabsisic acid; Olchemlm cat# 034 2721), d-IAA([2H5] indole-3- acetic acid; Olchemlm cat# 0311531, and d-JA (2,4,4-d3; acetyl-2,2-d2 jasmonic acid; CDN Isotopes cat# D-6936) and d-SA (d6- salicylic acid; Sigma cat#616796) was added to 100 mg ± 10 mg of ground tissue that were previously stored at -80^{o}C. The samples were then placed on a covered 4^{o}C shaker to prevent degradation by light and mix the samples. After 30 min, 500 µl of dichloromethane was added to the samples and placed back on the shaker for 30 min. The samples were then centrifuged for 5 min at 13,000 rpm, and the lower layer of the solution aliquoted into glass vials and evaporated with nitrogen gas. Samples were re-suspended in 150 µl of methanol and transferred to 1.5 ml microcentrofuge tubes. Samples were left overnight at -20^{0}C to allow debris to settle. Samples were then centrifuged at 14,000 rpm for 2 min to further remove the debris. Ninety µl of supernatant was placed into auto-sampler vials for direct injection into LC- (-)-ESI-MS/MS. The concurrent detection of hormones was utilized the methods of Muller and Munne-Bosch, 2011 with modifications. For quantification, the same

column and detector were used as stated above with a 600 µl/min mobile phase consisting of Solution A (0.05% acetic acid in water) and Solution B (0.05% acetic acid in acetonitrile) with a gradient consisting of (time-%B): 0.3- 1%, 2- 45%, 5-100%, 8-100%, 9-1%, 11-stop.

RNA Isolation and Quantification

Leaves were harvested at designated times after infection or exposure to volatiles and ground to fine powder using liquid N2. Samples were stored at -80^{0}C. Twenty to one hundred mg of the tissue was placed into 2 ml centrifuge tubes with 1 ml of TRI reagent (Molecular Research Center, Inc) and vortexed till fully suspended. Samples were stored at room temperature for 5 min. Next, 200 µl of chloroform was added to the samples to begin RNA extraction. Samples were inverted several times to mix the reagents and stored at room temperature for 15 min. Samples were then centrifuged in 4^{o}C at 12,000 rpm for 15 min. To precipitate the RNA the supernatant was transferred into 1.5 µl tubes and 500 µl of isopropanol was added. The samples were vortexed and left at room temperature for 10 min. Afterwards, samples were centrifuged in 4^{o}C at 12,000 rpm for 8 min. The supernatant was removed to leave a RNA pellet and washed with 500 µl of 75% ethanol in 0.1% Diethyl pyrocarbonate (DEPC) water. Next the samples were centrifuged at 8,000 rpm for 5 min. The supernatant was removed, and the RNA was washed 2 more times. The pellets were air dried in a fume hood to remove any leftover ethanol and the RNA was solubilized with 50 µl of 0.1% DEPC water. Next the samples were incubated in a 55-60^{o}C water bath for 5 min and placed on ice for 30 min. The RNA concentration was measured using spectroscopy (NanoDrop, Cole Palmer, Vernon Hills, IL, USA).

RNA was diluted to 125 ng/µl in 8 µl of 0.1% DEPC water and treated with DNAse (Thermo Scientific, cat# FEREN0521). The 8 µl of RNA was added to a RNase-free microfuge

tube along with 1 µl of 10x reaction buffer with $MgCL_2$, and 1 µl of DNase I, RNase-free (#EN0521). Samples were incubated at 37^{o}C for 30 min and then 1 µl of 50 mM EDTA was added. Lastly, samples were incubated at 65^{o}C for 10 min to inactivate the DNAse and stored at -20^{o}C.

Thermo Scientific Verso One-Step RT-qPCR Kits (Thermo Scientific, Waltha, MA, U.S.A) were used for quantitative reverse transcription-PCR (Q-RT-PCR) analysis. Each reaction was optimized to a volume of 10 µl consisting of 40 ng of DNase-free RNA and 200 nM primers. An Applied Biosystem StepOne Plus Real-Time PCR instrument was used for QRT-PCR analysis. The PCR program consisted of a 15-min cDNA synthesis step at 50°C. Next was the polymerase activation step of 15 min at 95°C, followed by 40 cycles of 30s at 54°C. The last step consisted of the melt curve analysis. Primers were designed using Primer3Plus software in accordance with the criteria required for quantitative PCR primer design (Udvardi et al., 2008). Absence of contaminating genomic, specificity DNA primers and lack of primerdimer formation was verified, using PCR analysis of RNA samples before reverse transcription, amplicon dissociation curves, and PCR in the absence of cDNA. LinReg (11.0) was used to test amplification efficiency of primers. Alpha-tubulin was used for the reference gene based on its stable expression for all treatments. Expression levels were normalized using alpha-tubulin and relative gene expressions was measured as fold change between mocktreated and PIPV-treated receivers (Ruijter et al., 2009).

Data Analysis

The statistical package JMP Pro 12 (SAS Institute, Inc) and excel were used for data analysis. Excel was used to perform t-tests under a null hypothesis to determine if two sets of data

were significantly different from each other. This type of test was used to determine the difference between lesion area, ergosterol, specific GLV content, and xylem sap content of plants treated with PIPVs or healthy plant volatiles (Figure 6, 7, 9, 12, and 15) A T-test was also used to compare FAW and *C. graminicola* plant induced volatiles effects on receiver susceptibility (Figure 16, and 17). Asterisks indicate a significant difference (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). Using JMP Pro 12 statistical package the effects of WT PIPVs on WT and mutant receivers, as well as *lox10* PIPVs on WT receivers were examined via ANOVA. Results that were shown to be variably significant were further analyzed using Tukey's HSD (Figure 10, 11, and 14).

RESULTS

PIPVs Induce LOX5 Expression in Receiver Plants

In previous research, former graduate student, Dr. Young-Soon Park, accidentally found that *LOX5*, a gene mediating susceptibility of maize to *C. graminicola* was unexpectedly induced in mock-treated plants grown near infected plants (Park, Unpublished). Because *LOX5* is a susceptibility gene for ALB (Park et al., 2010), this fortuitous result prompted my central hypothesis that volatiles emitted from infected plants induce *LOX5* expression in neighboring plants eventually resulting in increased susceptibility to *C. graminicola*. WT receiver plants were exposed to infected or mock treated emitter plant volatiles over a period of four days. Emitter plants were spray inoculated with *C. graminicola* or sterile water (mock-inoculated) and incubated for 24 h. The receivers were then exposed to the emitters for 24, 48, 72, and 96 h. qPCR analysis showed that receiver plants exposed to infected emitter volatiles displayed an exponential induction of the *LOX5* transcript accumulation with induction levels ranging between a 1.5-fold change at 24 h and a12-fold change at 96 h compared the receivers exposed to mock-treated emitters (Figure 5). These results suggest that while *LOX5* is not expressed in significant amounts in unchallenged leaf tissues (Park et al., 2010), it is strongly induced in the receivers exposed to volatiles from infected emitters, and suggest a relevance of this gene to host status of receivers.

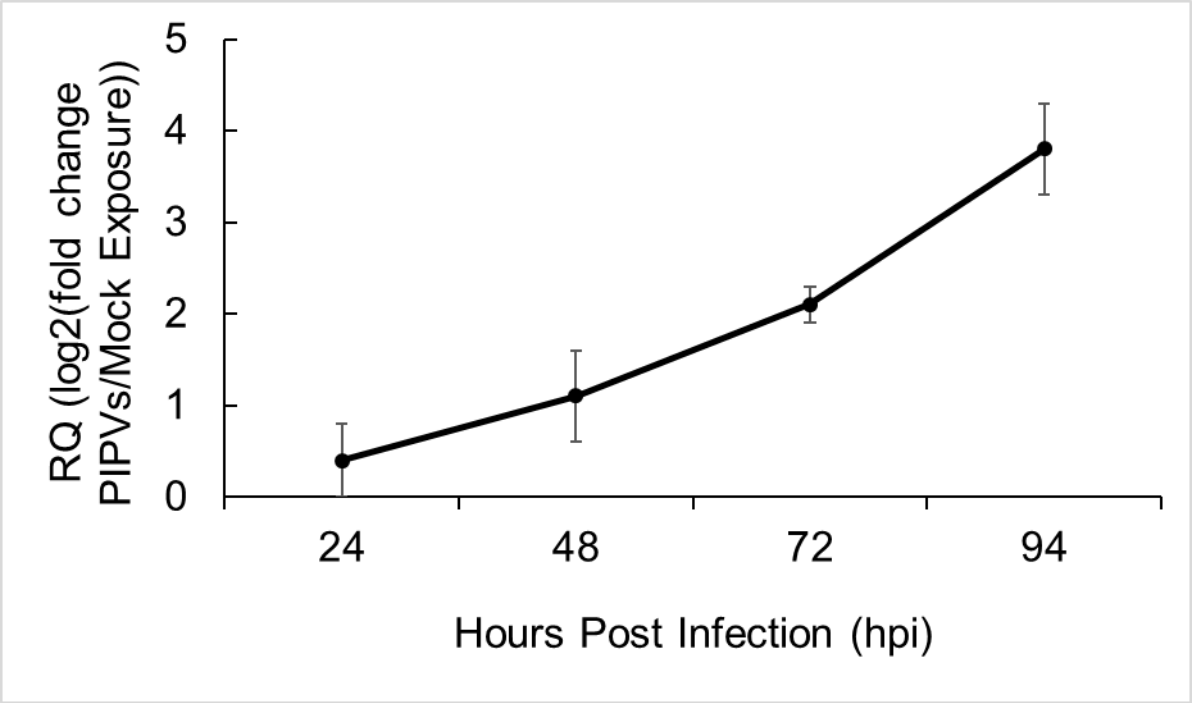

Figure 5: Increased relative expression of LOX5 in receiver plants exposed to PIPVs. Emitter plants were infected with *C. graminicola* or treated with sterile water (control). Receivers were exposed to emitters over a period of 4 days. The data are shown as mean log2 (fold change) ± SE.

PIPVs Predispose Neighboring Plants for Greater Susceptibility to Fungal Pathogens

To test the effect of PIPVs produced by infected emitters on the host status of receiver plants, receiver plants were exposed to the VOCs from emitters that were infected either by *C. graminicola* or *C. heterostrophus* for 48 h or mock treated. An infection period of 48 h was chosen because preliminary research showed that emitter plants were sufficiently infected by this time to produce PIPVs and elicit the greatest level of susceptibility in receivers. Volatiles from unchallenged emitters did not promote receiver susceptibility, however, emitters infected with *C. graminicola* or *C. heterostrophus* significantly predisposed receivers to pathogen infection (Figure 6). Receiver plants exposed to *C. graminicola* infected emitters were 10 times more susceptible than plants exposed to mock treated emitters as evidenced by significantly

larger lesion area (Figure 6A and B). The greater susceptibility due to PIPV was further confirmed by assessing fungal biomass as measured by leaf content of a fungal specific lipid, ergosterol, in infected receiver leaves. Receivers exposed to *C. graminicola* infected emitters had 8 times more ergosterol content when compared to mock exposed receivers (Figure 6C). Similar results were observed in receivers exposed to *C. heterostrophus* infected emitters, but to a lesser extent. Lesion areas of *C. heterostrophus* PIPV exposed receivers were 1.5 times larger (Figure 6 D and E) that corresponded to 1.3 times greater ergosterol levels (Figure 6F) when compared to receivers exposed to mock-treated emitters. These results demonstrate that *C. graminicola* and *C. heterostrophus* infected emitters produce volatiles that predispose receivers to become more susceptible to infection.

To determine if this induced susceptibility phenomenon is common in other maize lines, two popcorn lines, I29 and HP301, were chosen to be tested. Both lines showed increased susceptibility after PIPV exposure to *C. graminicola* (Figure 7). Lesion areas for the I29 line exposed to infected plant volatiles were 3 times larger when compared to receivers exposed to mock treatments and had 2.3 times more ergosterol content (Figure 7A and B). HP301 inbred displayed similar results by having 2.5 times larger lesion areas and 3 times more ergosterol content (Figure 7C and D). These results suggest that PIPVs induce systemic susceptibility (ISS) is consistent across several maize lines.

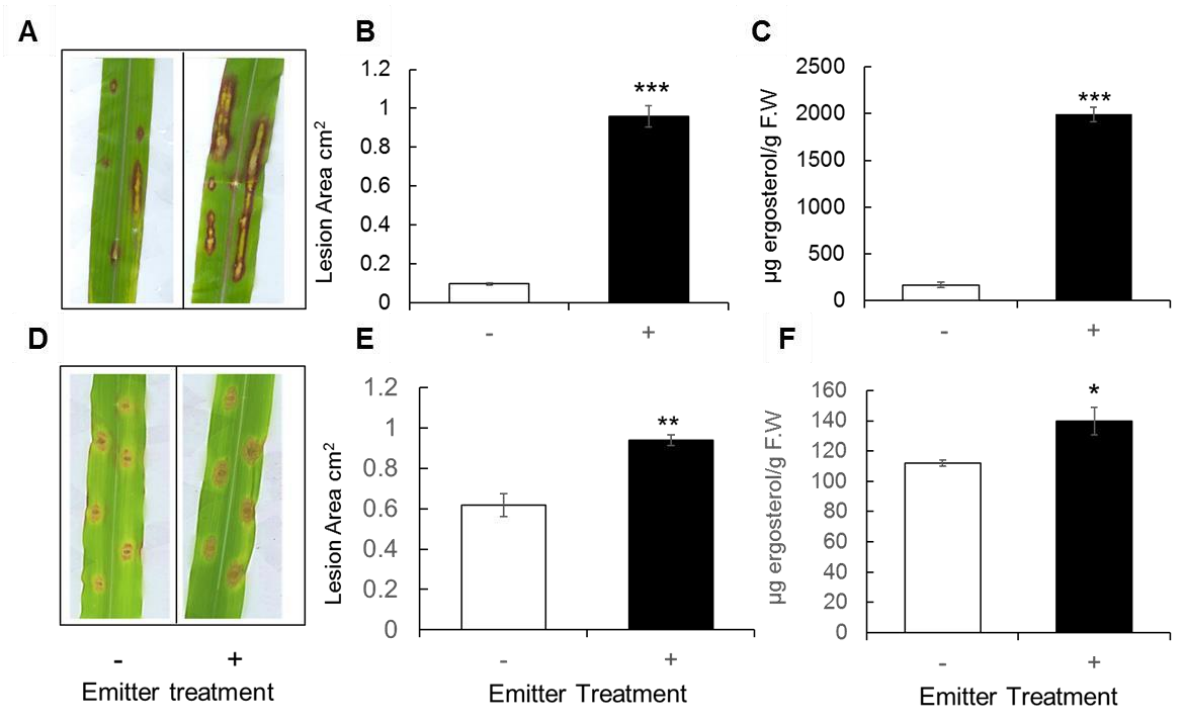

Figure 6: PIPVs induce susceptibility in exposed receiver plants. WT receiver plants infected with **(A)** *C. graminicola* infection or **(D)** *C. heterostrophus* after exposure to PIPVs (+) or control (-) VOCs. Lesion area (cm^2) of **(B)** *C. graminicola* and **(E)** *C. heterostrophus* infected plants after exposure to emitters. Ergosterol (µl/g F.W.) of **(C)** C. *graminicola* and **(F)** *C. heterostrophus* lesions after exposure to emitters. Emitter treatments were sterile water as the control (-) or spray inoculated by respective pathogens (+). Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

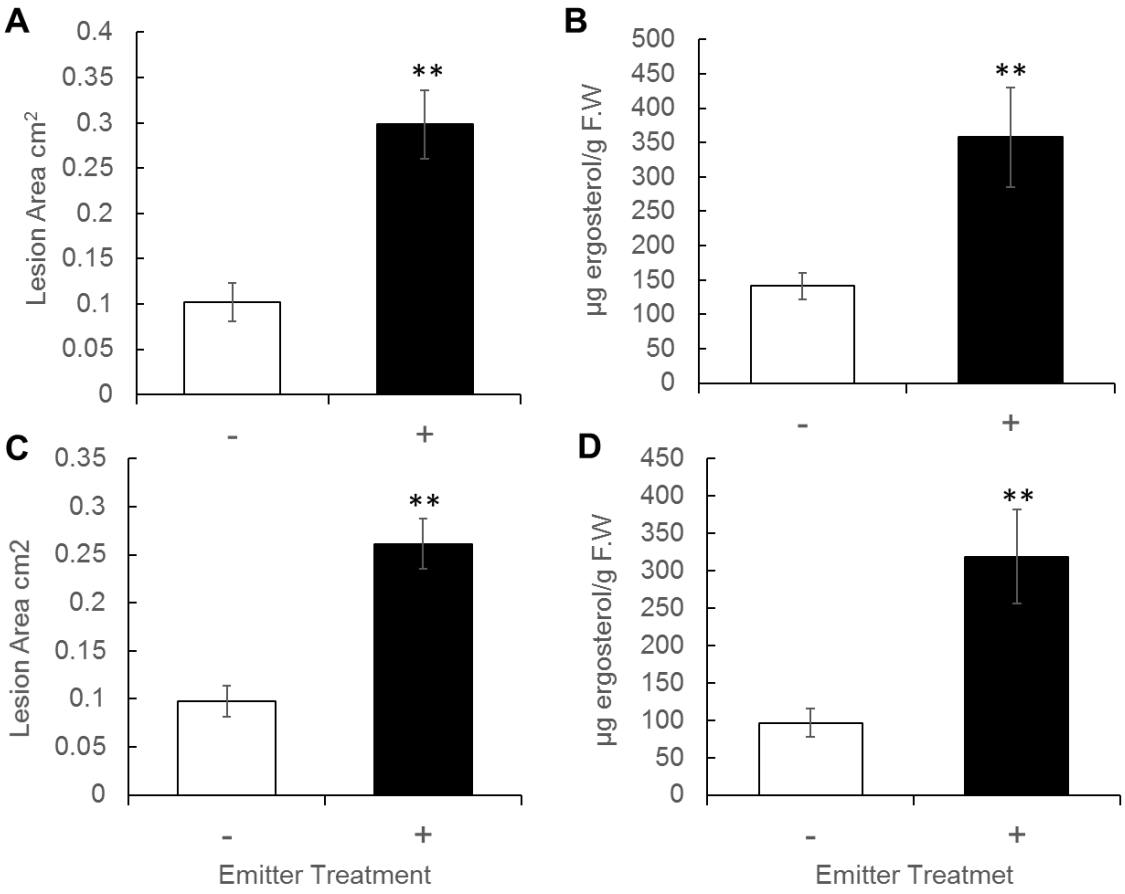

Figure 7: PIPVs induce susceptibility in receiver lines exposed to infected emitters. I29 and HP301 receiver plants were infected with *C. graminicola* after exposure to 48 h PIPVs (+) or control (-) VOCs. Lesion area (cm^2) of the infected **(A)** I29 and **(C)** HP301 receivers. Content of fungal specific membrane lipid ergosterol in infected **(B)** I29 and **(D)** HP301 measurement of lesions after exposure to infected or mock-treated emitters. Emitter treatments were sterile water as the control (-) and spray inoculation (+). Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

PIPVs Induce a Dramatic Transcriptome Reprograming in Receiver Plants

PIPVs not only induce systemic susceptibility in receiver plants, they elicit an enormous shift in the receiver transcriptome. Data was analyzed by comparing transcript count of PIPV and healthy plant volatile exposed receivers to reveal a significant increase in the accumulation of 2,052 and a decrease of 7,132 transcripts in PIPV exposed receivers compared to receivers

treated by volatiles from mock controls. Figure 8 depicts the greatest log2 transformed fold change of expression in the receivers exposed to PIPV. Induced transcripts included several heat shock proteins (HSP) along with their heat shock factors (HSF), multiprotein bridging factor 1 (MBF1), natural resistance-associated macrophage protein (Nramp1), and Caleosin related protein.

HSP are a subset of a larger group of proteins that act as molecular chaperones, which facilitate the synthesis and folding of proteins during times of stress. They have also been shown to participate in protein assembly, export, turn-over and regulation (Sørensen et al., 2003). HSP are mainly thought to be activated by abiotic factors such as drought, cold, and heat. However, they have been shown to be involved in the defense against pests, such as aphids and nematodes (Bhattarai et al., 2007). Arabidopsis MBF1c was induced in response to H_2O_2, ABA, SA, pathogen infection, salinity, drought and heat**.** When MBF1c is constitutively expressed in Arabidopsis, it exhibits enhanced tolerance to bacterial infection, osmotic stress, and heat (Miller et al., 2008). Nramp1 belongs to a highly-conserved eukaryote/prokaryote protein family. In mammals, Nramp1 regulates macrophage activation and is associated with infectious and autoimmune diseases. It has also been shown to be a bivalent cation (Fe^{2+}, Zn^{2+} and Mn^{2+}) transporter (Goswami et al., 2001). Caleosins are a family of lipid-associated proteins that are ubiquitous in fungi and plants. Caleosins have mainly been studied for their peroxygenase activity and have been shown to be upregulated following exposure to biotic and abiotic stresses (Partridge and Murphy 2009).

With the induction of HSP, HFS, MBF1, Nramp1, and caleosin related protein after PIPV exposure, receivers appear to be under stress. With the induction of stress related transcripts, several developmental related transcripts, such as glycosyl hydrolases family 16

and Vps52/Sac2 family were suppressed. Glycosyl hydrolases family 16 is involved in the biosynthesis of cell-wall polysaccharides. The cell wall plays a crucial role in growth, development, signal transduction, and cellular responses to environmental factors (Keegstra and Raikhel 2001). Vps52/Sac2 is required for pollen tube elongation, is localized in Golgi, and is involved in vesicle trafficking (Lobstein et al., 2004). Taken together, RNAseq analysis shows that PIPV exposed plants are under extreme duress and display substantial transcriptome reprograming their transcriptomics from growth processes to stress responses.

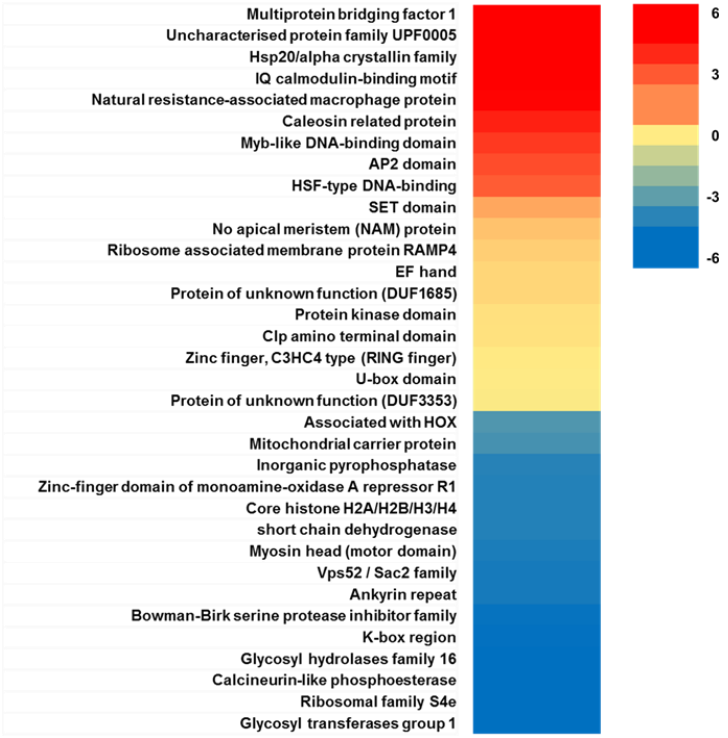

Figure 8: Heat-map showing induction or suppression of the expression of selected genes in response to PIPV treatment. The color scale is based on the log2 value of the fold-change of the PIPV libraries compared to the control treatment libraries. Log2 value = log2 (receivers exposed to PIPVs library/receivers exposed to control volatiles library). The red color indicates that the transcripts were more abundant in the PIPV exposed plants, while the blue color indicates that the transcripts were more abundant in the control treatment libraries.

GLVs Makeup a Fraction of the Total PIPV Content, but are Responsible for Inducing ISS

To determine what specific volatiles, constitute the PIPV blend that underlie ISS, a non-targeted analysis of the volatiles emitted by infected and mock plants was performed. Volatiles were collected for 3 h via a SuperQ from mock plants or plants that had been infected for 48 h with C. graminicola. Out of an estimated 1,000 volatiles detected, 30 volatiles were found to be emitted only by infected plants or had higher peak areas when compared to mock plants (Table 1). Of those thirty volatiles, three were GLVs, (Z)-3-Hexenal, (Z)-3-Hexenol, and (Z)-3-Hexenyl acetate, and they were chosen for further quantification. Further focus was on GLVs because of availability of knockout mutants in the GLV biosynthesis pathway (Christenson et al., 2013) which enabled me to address the question on whether GLVs mediate ISS. Figure 9A shows that infected plants produced significantly greater levels of GLVs than their mock counterparts. Specifically, infected plants emit double the amount of (Z)-3-Hexenal and (Z)-3-Hexenol and 3.5 times more (Z)-3-Hexenyl Acetate.

To determine if exogenous (Z)-3-Hexenal and (Z)-3-Hexenyl acetate could increase susceptibility in receivers, plants were exposed to biologically relevant concentrations of the synthetic volatiles based on the infected plant levels presented in Figure 9A. (Z)-3-Hexenal and (Z)-3-Hexenyl acetate concentrations were 2,000 and 4,000 fmol/h, respectively the results showed that both GLVs induced a significant increase in susceptibility compared to the control (Figure 9B). Infected plants were 9 or 13 times more susceptible based on ergosterol content, when treated with (Z)-3-Hexenal and (Z)-3-Hexenyl acetate, respectively, compared to control levels. These results suggested that GLV emission from infected plants maybe the volatiles responsible for promoting disease progression in neighboring plants.

Table 1: Volatiles produced by C. graminicola infected WT maize plants. Volatiles were collected via a SuperQ and analyzed using GC-MS.

Pathogen Induced Plant Volatiles	
1-chloro-dodecane	3-Ethyl-3-methylheptane
(E,E)-2,4-Heptadienal	4,5-dimethyl-nonane
(Z)-3-Hexenal	6-methyl-5-Hepten-2-one
(Z)-3-Hexenol	bis(2-ethylhexyl) ester Hexanedioic acid
(Z)-3-Hexenyl Acetate	butyl ester Acetic acid
1,2,4-trimethyl-benzene	Cyclobutyl ester-3-methyl-2-butenoic acid
1,2-dichloro-benzene	Cyclohexylmethyl heptadecyl ester sulfurous acid
1,4-Butanediol	Hexanoic acid
1-ethyl-4-methyl-benzene	Hexylene Glycol
2-(2-ethoxyethoxy)-Ethanol	Methyl salicylate
2,2-dimethyl-3-methylene-, (1S)-bicyclo[2.2.1]heptane	mono(2-ethylhexyl) ester 1,2-Benzenedicarboxylic acid
2,4,4,6,6,8,8-Heptamethyl-1-nonene	n-Butyl ether
2,4,4,6,6,8,8-Heptamethyl-2-nonene	Nonanoic acid
2,6-dimethyl-Cyclohexanol	Pentyl ester acetic acid
2-methylene-pentanedinitrile	Propanoic acid

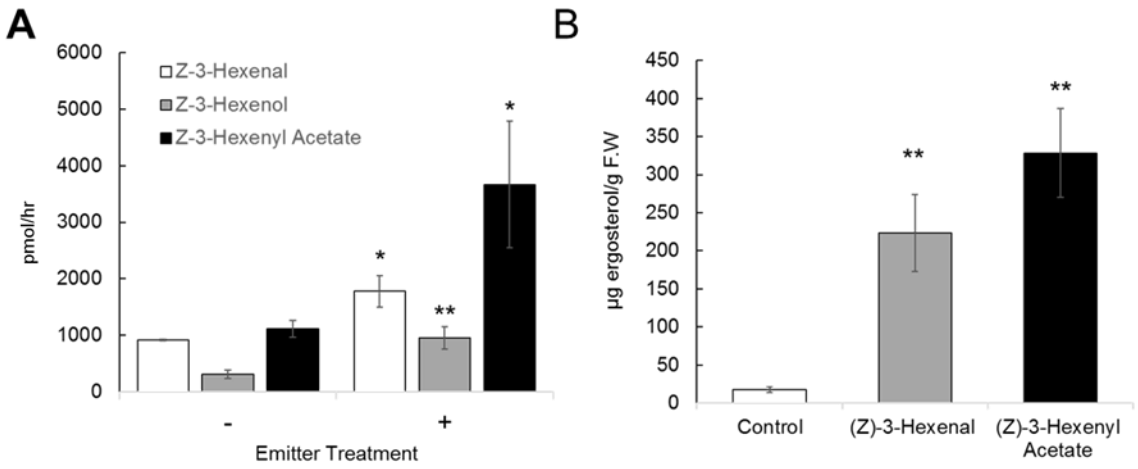

Figure 9: *C. graminicola* infected plants produce greater levels of Z-3-Hexenal and Z-3Hexenyl acetate that can induce susceptibility in exposed plants. **(A)** Volatiles were collected from two-day old WT plants spray inoculated with *C. graminicola* (10^6 spores/ml) (+) or sterile water (-). **(B)** WT plants were exposed to biologically relevant levels of GLVs, based infected plant GLV concentrations found in **A,** and drop inoculated with *C. graminicola* (10^6 spores/ml). Ergosterol was measured 7 days after infection. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

LOX10, the Sole Producer of GLVs, is Necessary for Infected Emitter Plants to Induce Susceptibility in Receiver Plants

In maize, LOX10 is the sole producer of GLVs in maize leaves, disruption of which results in plants being completely devoid of GLV emission (Christensen et al., 2013). To test the hypothesis that GLVs are principally responsible for receiver ISS, *lox10-3* mutant seedlings were used as GLV-deficient emitters and compared to near-isogenic WT. As in previous experiments, seedlings were inoculated with *C. graminicola* or mock-treated for 48 h and then exposed to WT receiver plants for 3 h followed by inoculation. Figure 10 shows that unlike WT plant emitters, which promoted susceptibility in neighboring plants, GLV-deficient emitters did not induce ISS in receivers as measured by the lack of increased lesion area and fungal biomass (Figure 10A and B). These data provide genetic evidence that GLVs emitted by infected emitters are necessary for inducing ISS in neighboring plants.

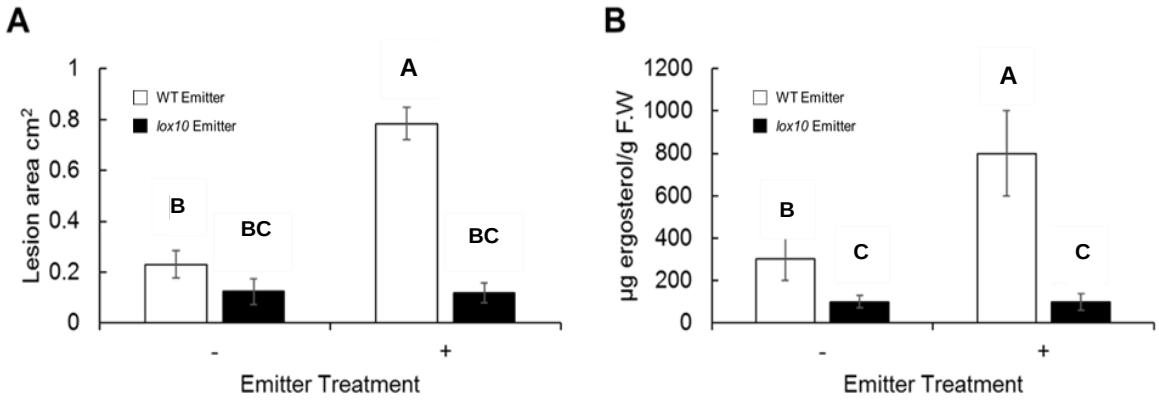

Figure 10: LOX10 and GLV emission is necessary in infected emitter plants to promote susceptibility in receiver plants. WT and *lox10* emitter plants were inoculated with *C. graminicola* or sterile water (control) and disease was allowed to progress for 48 h. Afterwards WT receivers were exposed to the infected (+) or mock-treated (-) emitters for 3 h, after which they were drop inoculated by *C. graminicola*. After disease development ergosterol was extracted. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

PIPV-Treated Emitters Display Increased Expression of JA Biosynthesis Genes and Increased Accumulation of JA

JA is a well-studied insect defense hormone. Recent unpublished data indicates that JA-deficient *opr7opr8* double mutants are remarkably more resistant to *C. graminicola* due to increased levels of pathogen-induced SA (Yan, He, Gorman, Kolomiets, unpublished) suggesting that JA may facilitate pathogenesis by this hemibiotrophic pathogen. To determine if the ISS observed in PIPV exposed receivers is caused by an increase in JA, phytohormone analysis was performed on leaf tissue of WT and *lox5-3* receivers (Figure 11). *lox5-3* mutants were chosen as receivers because preliminary research indicated that they did not response to PIPVs with ISS. The WT and *lox5-3* receivers were exposed to PIPVs or healthy emitter volatiles for 3 h. The results demonstrated that PIPV exposed WT receivers had elevated levels of both JA and JA-Ile, the active form of JA, when compared to receivers exposed to VOCs from non-infected emitters (Figure 11). Average JA levels in WT receiver leaves exposed to PIPVs were 2.7 times higher than mock exposed WT receivers and *lox5-3* receivers (Figure 11). The levels of JA-Ile in PIPV exposed WT receiver leaves were on average 3.5 times higher than all other receivers tested (Figure 11). Hormones such as JA are transported from the roots to the leaves of a plant via xylem sap. Since JA was shown to be increased in PIPV exposed receiver leaves, the hormone composition of the receiver xylem sap was analyzed. Both JA and JA-Ile hormone levels in xylem sap were significantly elevated in PIPV exposed receivers compared to mock exposed receivers (Figure 12). Taken together, these data illustrate that JA is indeed increased in receivers exposed to PIPVs and maybe responsible for ISS.

After the discovery of elevated JA and JA-Ile levels in PIPV exposed WT receivers, JA regulation and biosynthesis genes were analyzed in emitters and receivers. Of the maize genes

tested, *LOX8, AOS1a,b,c, AOC2,* and *OPR7* are all directly involved in the biosynthesis of JA while *MYC7* is the transcription factor of JA. Though not directly involved in JA biosynthesis *LOX5* and *LOX12* are involved in JA regulation by producing yet to be identified 9-oxilipins with potent hormone-like activities (Borrego and Kolomiets, 2016). Expression of GLV biosynthesis genes, *LOX10* and *HPL1,* were also measured. To determine the expression of these JA-related genes in emitter plants, plants were treated with conidia of *C. graminicola* or water (control), incubated for 48 h and then leaf tissue was collected. During an emitter infection, JA related genes were not induced in response to infection (Fig. 13A). In contrast, when receiver plants are exposed to volatiles from infected plants, JA related genes were significantly induced compared to plants exposed to volatiles from healthy, mock-treated emitters (Figure 13). Interestingly, although infected emitter plants do not display an induction of JA related genes, expression of the GLV-producing *LOX10* gene was increased by 36-fold in infected plants compared to control plants (Figure 13A). The induction of *LOX10* further supports that the notion that GLVs are massively produced by infected plants and may be responsible for increased susceptibility of neighboring receiver plants.

To gain insight as to what are the genes in the receiver plants that may underlie ISS, expression of the same set of GLV and JA-relevant genes were assessed in the receivers treated with PIPVs or healthy plant volatiles for 3 h. The results showed that transcript levels of the *LOX5, LOX8, LOX12, AOS1b, OPR7,* and *MYC7* genes are significantly increased when compared to receiver plants exposed to volatiles from mock-treated emitters (Figure 13B). Specifically, expression of *LOX5* was increased by 7.5-fold. This data further supports the results that *LOX5* is induced by PIPVs as presented in Figure 5. Similar to *LOX5*, *LOX12* is not directly involved in JA production, but is essential for JA induction in response to infection

by *Fusarium verticillioides* (Christensen et al., 2014). In receiver plants exposed to PIPVs, *LOX12* transcript levels were increased 21 times compared to treatment with volatiles from healthy plants. *LOX8, AOS1b,* and *OPR7,* which are directly involved in JA biosynthesis, displayed 18, 24, and 18-fold increase in relative expression, respectively. *MYC7* is a transcription factor that is responsible for the production of JA inducible genes. *MYC7* transcripts were induced 15-fold by PIPVs. In contrast to infected emitters (Fig. 13A), receivers exposed to PIPVs did not express GLV biosynthesis genes, *LOX10* or *HPL1*, suggesting that GLVs are not induced in receivers. Taken together, these results show that JA, a susceptibility hormone for *C. graminicola,* is increased in receivers exposed to infected emitters and is the most likely candidate for causing ISS.

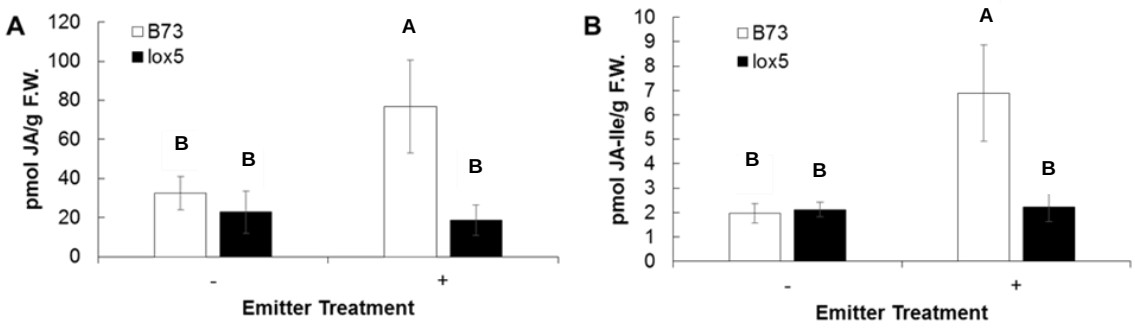

Figure 11: JA and JA-Ile levels in leaf tissues are increased in PIPV exposed WT receivers. WT and *lox5* receiver plants were exposed to 48 h *C. graminicola* infected (+) or mock (-) receivers, after which they were drop inoculated with conidia of *C. graminicola*. The third leaf of each receiver was used for hormone analysis. **(A)** Depicts increased JA levels in PIPV exposed WT receivers. **(B)** Shows elevated JA-Ile levels in PIPV exposed WT receivers. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

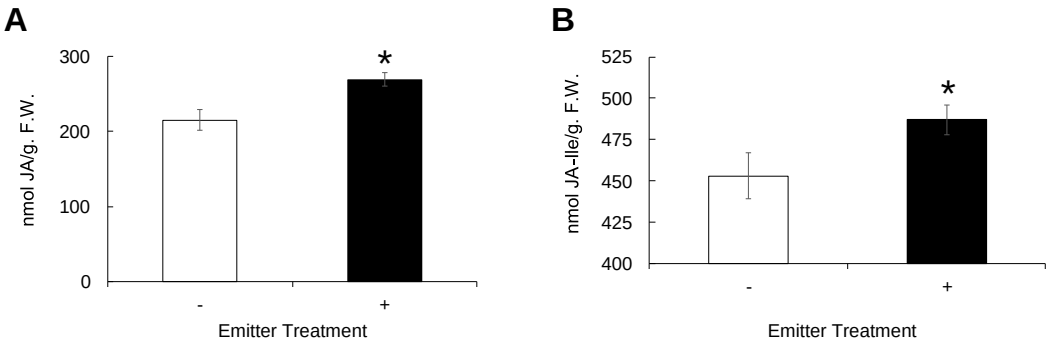

Figure 12: JA and JA-Ile levels are elevated in PIPV exposed receiver sap. Receivers were exposed to PIPVs (+) and mock (-) volatiles for 3 hours and xylem sap was extracted for 3 h. **(A)** Depicts increased JA levels in PIPV exposed receivers. **(B)** Shows elevated JA-Ile levels in PIPV exposed receivers. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

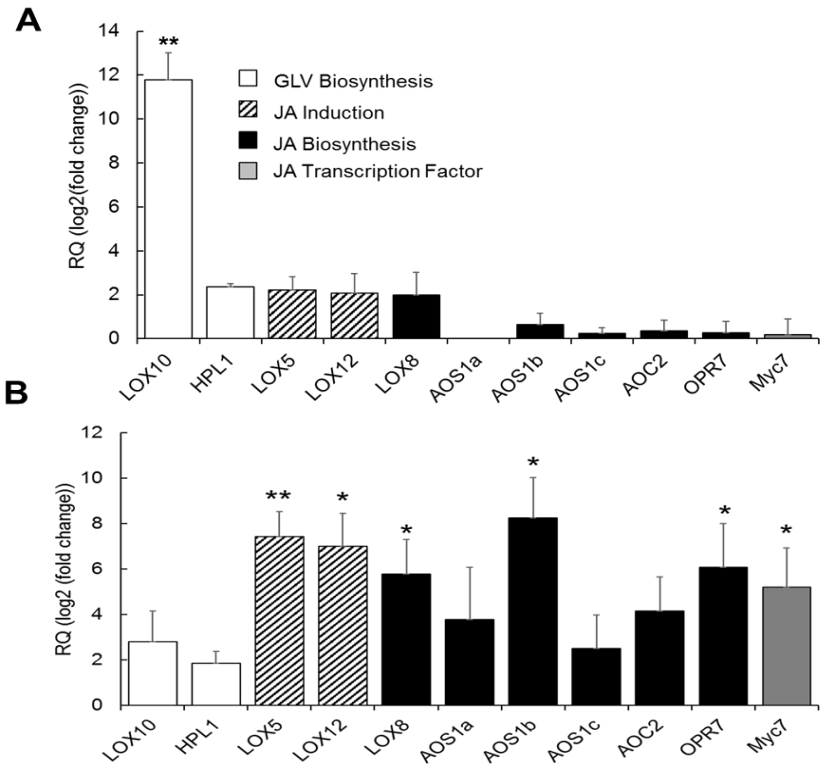

Figure 13: Expression of the GLV and JA related genes in infected emitters and receivers exposed to infected emitters. (A) Gene expression in emitter plants infected for 48 h with *C. graminicola* (10^6 spores/ml). (B) Gene expression in receiver plants exposed to infected vs mock-treated emitters for 3 h. The data are shown as mean log2 ± SE with PIPV-treated plants compared to mock-treated plants and only those means with statistically significant change in

gene expression are designated with an asterisk. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Functional LOX2, LOX5, and LOX12 are Essential for Predisposing PIPV Exposed Receivers for Greater Colonization by *C. graminicola*

After establishing which genes were induced in the receivers by PIPV exposure, available transposon-insertional LOX and OPR mutants were used as receivers to test if those genes were necessary for the induction of susceptibility in the plants exposed to infected emitters. The mutant alleles tested included *lox1-3*, *lox2-1*, *lox5-3, lox12-1*, *opr2-1*, and *opr75*, because the respective genes were either induced by treatment with PIPVs (Fig. 13B), or were shown in previous research to be GLV-inducible (*OPR2*, *LOX1, LOX2* and *LOX5*) (Engelberth et al., 2007; Park et al., 2010). WT emitter plants were inoculated for 48 h, after which, the mutant or WT receivers were exposed to the emitters for 3 h and infected with *C. graminicola*.

Of the mutant receivers exposed to healthy plant volatiles, *lox1-3,* and *lox2-1* were more resistant to *C. graminicola* after exposure when compared to WT receivers, while *lox53, lox12-1,* and *opr2-1* were significantly more susceptible (Figure 14A, C, D, E, and F). The *opr7-5* receivers did not display a significant difference in their susceptibility when compared to WT receivers (Figure 14B). After exposure to PIPVs only *lox2-1, lox5-3, lox12-1* and *opr21* knock-out alleles did not display increased fungal biomass in response to PIPVs, suggesting that these genes in the receiver plants are essential for predisposing plants for greater susceptibility (Figure 14C, D, E, and F). Of these genes, the 9-LOXs, *LOX5* and *LOX12*, have been shown to be involved in the positive regulation of JA biosynthesis (Park et al., 2010; Christensen et al., 2014). Although *LOX2*'s involvement in JA regulation and biosynthesis has not been well established, previous research has shown that JA synthesis in response to wounding is regulated by *LOX2* (Huang, unpublished). The finding that *OPR2* is also important in mediating ISS in

PIPV exposed receivers is surprising since *OPR2* is not predicted to be involved in JA biosynthesis as it belongs to the non-JA producing OPRI subfamily (Zhang et al., 2005). More research will have to be done to determine, if *OPR2* plays a role in JA regulation and biosynthesis. Interestingly, *lox12-1* and *opr2-1* both had a significant decrease in susceptibility after PIPV exposure when compared to their exposure to healthy plant volatiles. The reason for this decrease is not known, but it does clearly indicate that these genes are required for ISS.

Of the mutants tested, *lox1-3* mutants displayed increased susceptibility after PIPV exposure, indicating that even though this gene is GLV-inducible, LOX1 is not involved in ISS (Figure14 A). Like *lox1-3*, *opr7-5* mutants did not show a difference in their susceptibility levels when compared to the WT receivers after PIPV exposure (Figure 14F). However, *OPR7* is one of the two genes needed to reduce OPDA in the JA pathway (Yan et al., 2012). The other gene is *OPR8*. When one of these genes are disrupted, JA production is not reduced in any organ or by any treatment because the genes are able to compensate for each other (Yan et al., 2012). The ISS observed in *opr7-5* mutants could be due to the presence of functional *OPR8* as the two genes are redundant for JA biosynthesis. Further research will test whether JA-deficient *opr7opr8* double mutants retain ISS response after PIPV exposure. Taken together, these results demonstrate that LOX2, LOX5, LOX12 and OPR2 are essential to predispose greater colonization of receivers after PIPV exposure.

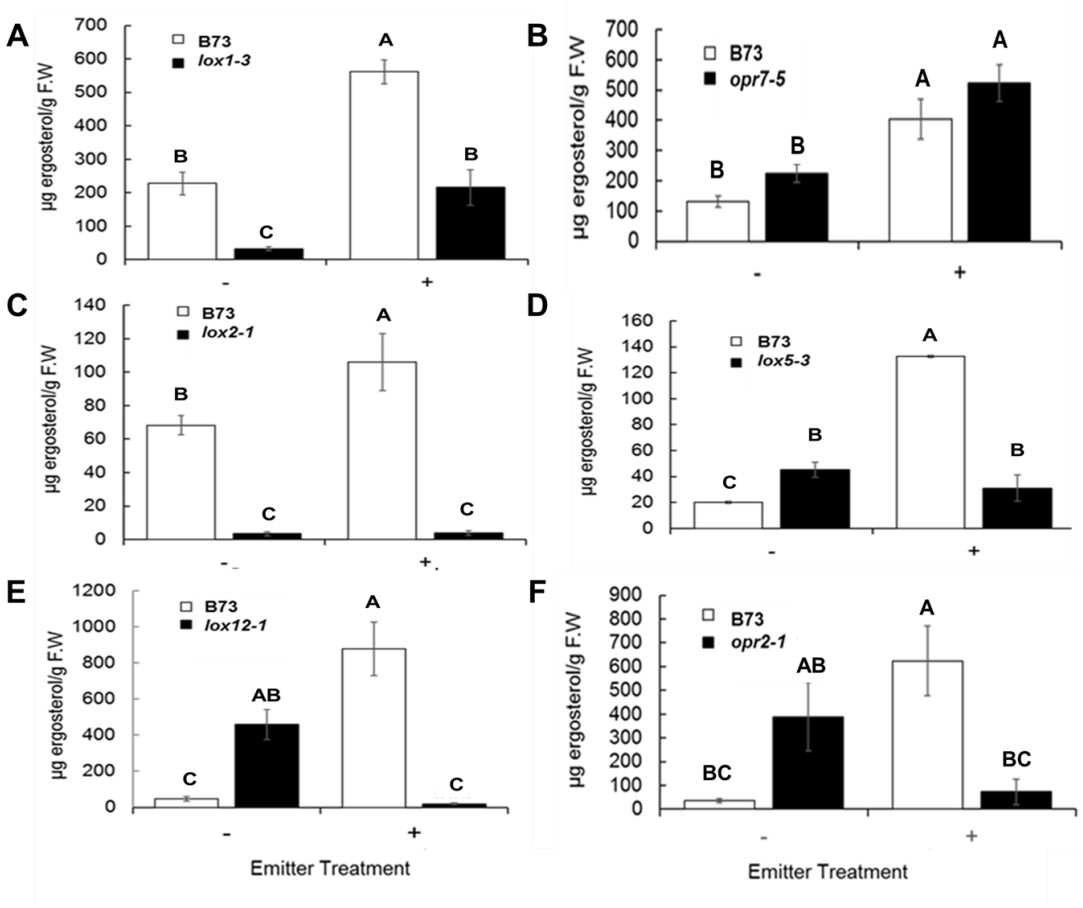

Figure 14: LOX2, LOX5, LOX12 and OPR2 are required for receiver susceptibility after exposure to PIPVs. WT emitter plants were infected with *C. graminicola* (10^6 spores/ml) and disease progressed for 48 h. After 48 h, the WT and mutant receivers were exposed to the infected (+) or mock (-) emitters for 3 h, after which they were drop inoculated with *C. graminicola*. WT receiver plants were compared to *lox1-2* **(A),** *opr7-5* **(B)**, *lox2-1* **(C)**, *lox5-3* **(D)**, *op12-1* **(E)**, and *opr2-1* **(F)** receiver plants. Letters above the bars indicate significant differences at $p < 0.05$.

PIPV Exposed Receiver Xylem Sap Increases *C. graminicola* Pathogenicity

To address whether PIPV exposed receiver xylem sap can induce ISS, xylem sap from the exposed receivers was introduced into unexposed plants to test their susceptibility. Receiver plants were exposed to 48 h infected or health plant emitters for 3 h. Afterwards xylem sap was collected from the receivers for another 3 h. As a control, sap was also extracted from unexposed plants. It should be noted that the sap used for this experiment is the same from the

hormone analysis in Figure 15. Xylem sap from the control, mock, and PIPV receivers was then applied to WT plants which were then challenged with *C. graminicola.* Application of xylem sap collected from PIPV exposed receivers elicited the same ISS observed in PIPV exposed receivers as evidenced by the greater lesion area and increased fungal biomass levels compared to both the control and mock sap (Figure 15). These results show that PIPV exposed receiver xylem sap leads to ISS, which could be caused by the elevated levels of JA observed in PIPV exposed plants.

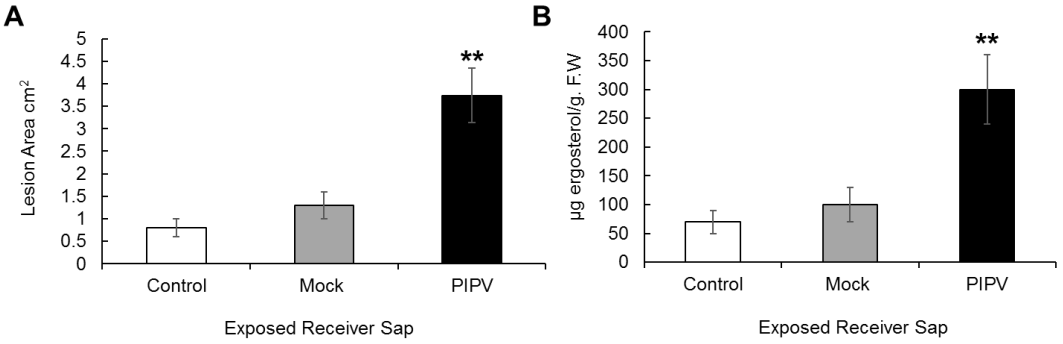

Figure 15: Effects of PIPV exposed receiver xylem sap on *C. graminicola* pathogenicity. Xylem sap extracted from mock and PIPV exposed receivers and unexposed plants was diluted 1:1 with sterile distilled water and injected into WT plants. The injected plants were then drop inoculated with *C. graminicola* and disease was allowed to progress for 5 days. **(A)** Lesion area of plants treated with control, mock, or PIPV exposed receiver sap and challenged with *C. graminicola.* **(B)** Fungal biomass. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

PIPVs and HIPVs Can Both Elicit Host Susceptibility to *C. graminicola*

During an insect attack, infested plants produced HIPVs that act as location cues for herbivore predators and aid in the defense of the infested plants (Furstenberg et al., 2013). GLVs, specifically (Z)-3-Hexenal and Z-3-Hexenyl acetate, are a significant part of HIPVs and are responsible for eliciting an induction of JA in exposed neighboring plants (Farag et al.,

2005, Ton et al., 2007). Data depict that PIPVs also contained (Z)-3-Hexenal and Z-3-Hexenyl acetate and can elicit an increase in JA levels. Taken together HIPVs can predispose plants to host susceptibility through the induction of JA. Receiver plants were exposed to mock, 48 h *C. graminicola* infected, and fall army worm (FAW) infested emitters for 3 h then inoculated. Both *C. graminicola* and FAW volatiles induce ISS in receivers, through PIPV exposure produced more susceptibility than HIPV (Figure 16). When compared to the mock exposed receivers, PIPV and HIPV exposure had 3.5 times and 2.5 times larger lesion areas respectively (Figure 16A). As for fungal biomass, PIPVs induced 7.5 times more fungal biomass while HIPVs produced 3 times more (Figure 16B). These results show that both HIPVs and PIPVs predispose hosts to susceptibility presumably through the emission of GLVs and subsequent JA induction in exposed plants. However, since PIPVs induced a greater response than HIPVs there is most likely some specificity volatiles involved.

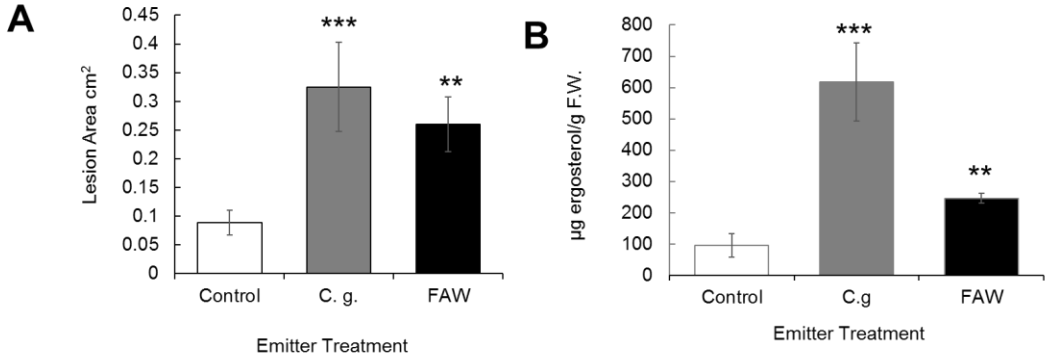

Figure 16: PIPVs and HIPVs induce ISS in exposed plants. Receivers were exposed to control, 48 h *C. graminicola* infected, and FAW infested emitters for 3 h. **(A)** Shows increased lesion area of receivers exposed to infected and infested emitters. **(B)** Depicts a greater fungal biomass in receivers exposed to infected and infested emitters. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

PIPVs do not Prime Neighboring Plants for Insect Defense

Given that HIPVs can induce ISS in neighboring plants, it was hypothesized that PIPVs could prime plants for insect defense. Receivers were exposed to control, infected, and infested emitters for 3 hours, then challenged with FAWs for 3 h. HIPVs exposed plants were significantly more resistant to insect attack as observed with a decrease in area consumed when compare to the control receivers (Figure 17). However, PIPVs were unable to prime exposed plants for insect defense (Figure 17). (E)-2-hexenal is a GLV that is emitted by infested plants, but not produced in infected plants. To produce and emit (E)-2-hexenal plants need the oral secretions of herbivores (Baldwin et al., 2010). This insect specific GLV may be the reason PIPVs cannot induce insect resistance in receiver plants, but HIPVs can.

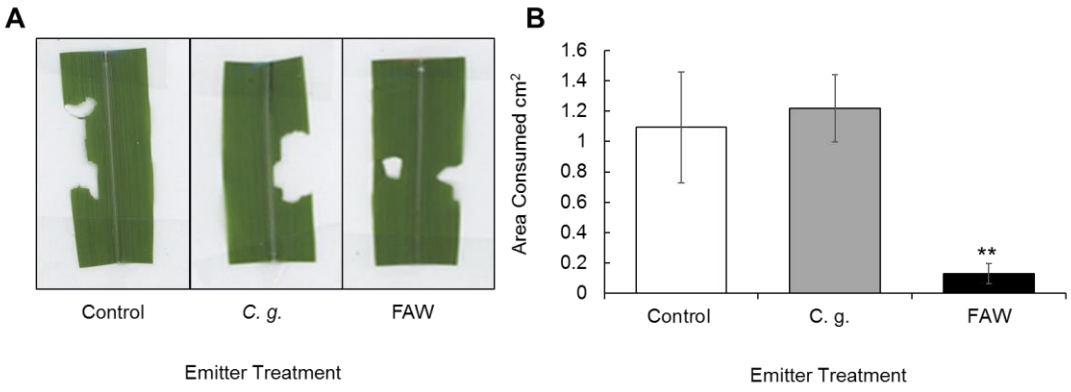

Figure 17: Unlike HIPVs**,** PIPVs do not prime plants for herbivory defense. Receivers were exposed to control, 48 h *C. graminicola* infected, and FAW infested emitters for 3 h. Afterwards they were challenged with FAWs for 3 h. **(A)** Representative images of receivers challenged with FAW after emitter exposure. **(B)** Depicts the receiver area consumed by FAWs. Statistically significant differences (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

SUMMARY

Numerous studies have shown that HIPVs are involved in intra-and inter-plant defenses against insects by eliciting the help of herbivore predators and priming JA biosynthesis in neighboring plants (Furstenberg et al., 2013; Christensen et al. 2013; Ton et al., 2007). Unlike HIPVs, PIPVs are not well studied, but have been demonstrated by this research to be involved in induced systemic susceptibility of the neighboring plants to at least two fungal pathogens tested by an induction of JA regulation and biosynthesis in receivers. This research originated from a serendipitous discovery that *C. graminicola* infected plants emit volatiles that were able to induce expression of the *LOX5* gene in plants grown in close proximity to infected plants. Further analyses revealed that *LOX5* is induced strongly by insect feeding (Park et al., 2010) and GLV exposure (Park et al., 2010). In agreement with insect defense relevant expression pattern, *lox5* knock-out mutants are substantially more susceptible to insect feeding (Park et al., 2010). This suggested that LOX5 is required for defense against chewing insects and their induction by GLV may be one mechanism of JA priming in the unchallenged neighboring plants. However, *lox5* mutants are remarkably more susceptible to *C. graminicola* suggesting that *LOX5* is a susceptibility gene that facilitates pathogenicity (Park, 2010). These results collectively prompted the central hypothesis of this study which is that volatiles emitted from infected plants induce *LOX5* expression in neighboring plants and this induction eventually results in increased susceptibility to *C. graminicola*.

Testing this hypothesis uncovered that maize seedlings exposed to infected neighboring plants were indeed more susceptible to subsequent infection by fungal pathogens, a phenomenon called induced systemic susceptibility or ISS. The results described here show that the ISS response occurs in three genetically diverse maize inbred lines, B73, I29 and

HP301, and in response to two major hemibiotrophic fungal pathogens, *C. graminicola* and *C. heterostrophus*, (Figure 6 and 7). This suggests that volatiles produced by infected plants may, more commonly than previously reported, predispose neighbor unchallenged plants for increased susceptibility to imminent pathogen infections. While volatiles produced by infested plants, HIPVs, have been widely known to induce systemic resistance to subsequent insect feeding, our results indicate that pathogens may manipulate volatile production for priming the neighbor plants for increased susceptibility. PIPV mediated ISS may provide a novel line of research for explaining the mechanistic basis for epidemics commonly occurring in fields under conducive environmental conditions. In a field setting, infected plants can prime neighboring plants to become more susceptible through volatile exposure.

While our results suggest that PIPVs are composed of over 1,000 different volatiles, our results have demonstrated that specifically GLVs are primarily responsible for induction of ISS. Of the GLVs produced by plants (Figure 3), synthetic (Z)-3-Hexenal and (Z)-3-Hexenyl acetate are potent priming signals for ISS response (Figure 9B). Providing genetic evidence for the idea that GLV are the primary volatiles inducing ISS, GLV-deficient *lox10* mutants were used as emitters and completely failed to illicit ISS in receiver plants (Figure 10). Interestingly, *LOX10* expression was significantly induced in *C. graminicola* infected emitter plants accompanied by increased GLV production (Figure 9A), but not in non-infected receivers exposed to PIPVs. making it tempting to hypothesize that GLV induction by pathogens may be a part of their pathogenicity strategy to prime neighboring plants to become a more suitable host for impending spore spread. Taken together, this indicates that GLVs produced by infected plants are responsible for inducing ISS in neighboring plants. This is in sharp contrast to GLVs

proven roles in priming nearby plants for greater defense against impending insect attack (Turlings et al., 2000).

To induce physiological responses including ISS, plants must first perceive the volatiles emitted by infected plants. To date, however, the exact mechanisms of GLV perception in plants has not been elucidated. Based on analogy to GLV perception in other organisms, it is reasonable to hypothesize that plants may perceive volatiles as ligands of G protein coupled receptors (GPCR) because both mammals and insects perceive GLVs by this group of cell receptors (Andersson et al., 2015, Vassilatis et al., 2003). GPCR, some of which are known as olfactory receptors (ORs), are seven transmembrane proteins that form the largest single family of integral membrane receptors that respond to odorants and give rise to the sense of smell in mammals (Gaillard et al., 2004, Ferguson et al., 2001). Through genome analysis,
56 putative GPCRs have been identified in plants (Taddese et al., 2014), but their identity as GPCRs remains unresolved.

Similar to HIPV emissions, PIPVs contain GLVs that serve as priming signals to induce JA biosynthesis in the receiver plants (Figure 13B). Interestingly, JA biosynthesis and regulation genes in plants infected for 48 h were not induced, indicating that JA is not produced during this infection period (Figure 13A). Of the JA related genes, *LOX5, LOX8, LOX12, AOS1b, OPR7*, and *MYC7* were significantly induced in PIPV-exposed plants. *LOX8, AOS1b* and *OPR7* genes are directly involved in the JA biosynthesis pathway. *LOX5* and *LOX12* are not directly involved in JA biosynthesis, but have been shown to play a role in positive regulation of JA production (Borrego and Kolomiets 2016). LOX5 produces a 9-oxylipin(s) that serves as a hormone-like signaling molecule essential for JA induction upon wounding

(Park et al., 2010), and *LOX12* is needed to mount a JA-mediated defense against a *Fusarium* infection in maize (Christensen et al., 2014). Phytohormone analysis of leaf tissue and xylem sap showed that PIPV-exposed plants had elevated JA and JA-Ile levels (Figure 11 and 12). Interestingly, xylem sap had higher concentration of JA and JA-Ile compared to leaves. When xylem sap extracted from PIPV-treated receivers was injected into plants before infection, they were more susceptible to *C. graminicola*, indicating that the JA-enriched xylem sap carries some long distance signaling molecules that suppresses defense against this pathogen. Corroborating the notion that JA is one such susceptibility signal, JA has been found to promote susceptibility to *C. graminicola*. JA-deficient *opr7opr8* mutants were highly resistant to anthracnose stalk rot, indicating JA is a susceptibility hormone (He and Yan, unpublished). Collectively these data show that the ISS response in exposed receivers is due to the induction of JA.

Several LOX and OPR genes are GLV inducible and are vital for the biosynthesis or signaling regulation of JA. Mutants of these genes were chosen as receivers to test their involvement in receiver ISS. Of the genes tested *LOX2, LOX5, LOX12* and *OPR2* were revealed to be vital for increased susceptibility in PIPVs-treated receivers (Figure 14). *LOX2* involvement in JA regulation has not been well studied, previous research has shown that *LOX2* regulates JA synthesis in response to wounding and is GLV-inducible (Park and Huang, unpublished). Interestingly, *LOX2* was not induced by PIPVs from infected emitters at 48 hpi (Figure 13B), suggesting that the concentration of (Z)-3-Hexenyl acetate in the PIPV blend was not sufficient to induce expression at that time point. *LOX5* is both GLV-and PIPV inducible and is involved in wound-induced JA biosynthesis (Park et al., 2010). Without the functional *LOX5*, PIPV exposed plants no longer respond to volatiles by increased

susceptibility, illustrating that *LOX5* is essential for ISS (Figure 14C). *LOX5* involvement in ISS was surprising because it was first described as an insect defense gene (Park et al., 2010). The next LOX gene that was necessary for ISS is *LOX12*. This gene is induced by PIPVs to the greatest level of all tested genes. *LOX12* is required for normal induction of JA biosynthesis in response to *F. verticillioides* infection. It is likely that the reasons these mutants are no longer susceptible to infection upon PIPV treatment is the lack of induction of JA by PIPVs as shown for the *lox5* mutants (Figure 11).

The finding that OPR2 is also important to mediate ISS in PIPIV exposed receivers is surprising since OPR2 is not predicted to be involved in JA biosynthesis as it belongs to the non-JA producing OPRI subfamily (Zhang et al., 2005). OPR7 and OPR8, are the only JA producing OPRs in the maize genome (Yan et al., 2012). Further investigation of whether *opr2* mutants are altered as well in JA biosynthesis in response to PIPVs will be required to place this gene in the context of ISS responses. The relevance to ISS of JA-producing OPR7 and OPR8 is not clear as single *opr7-5* mutant receivers displayed ISS (Figure 14F). This result is most likely due to functional *OPR8* compensating for the loss of *OPR7* mutant and allowing for normal biosynthesis of JA. To test *OPR7* and *OPR8* involvement in ISS, *opr7opr8* double mutants will be analyzed in the future.

Previous research on the virulence factor coronatine, a functional mimic of JA-Ile, produced by *Pseudomonas syringae* sets a precedence for the research preformed for this dissertation (Katsir., 2008). It should also be noted that like *C. graminicola, P. syringae* is a hemibiotroph. Once secreted by *P. syringae*, coronatine represses the host defense responses by activating JA signaling in a COI1-dependent manner which leads to increased susceptibility to the pathogen (Katsir eat al., 2008). Because both *P. syringae* and *C. graminicola* use JA

signaling to elicit host susceptibility it gives support to the conclusion that JA promotes susceptibility to *C. graminicola*.

Since HIPVs and PIPVs both contain GLVs, I reasoned that HIPVs could induce ISS to *C. graminicola* infection and, conversely, PIPVs could induce ISR to an insect attack. The results showed that HIPVs induced both ISS and ISR, as expected. However, PIPVs were only able to induce ISS regarding pathogen infection, but not ISR to insect feeding (Figure 16 and 17). Though HIPVs and PIPVs both contain (Z)-3-Hexenal, (Z)-3-Hexenol, and (Z)-3Hexenyl acetate, only HIPVs contain (E)-2-hexenal. (E)-2-hexenal has been shown to be only produced in infested plants due to insect oral secretion containing an enzyme for production of this molecule (Baldwin et al., 2010), but not in maize infected with *C. graminicola* (data not shown) Therefore I hypothesize that PIPVs cannot induce ISR to an insect infestation due to the lack of (E)-2-hexenal. I also reason that HIPVs may induce ISS to fungal colonization because they contain (Z)-3-Hexenal and (Z)-3-Hexenyl acetate, the two GLVs responsible for inducing susceptibility in neighboring plants as shown in Figure 9.

In conclusion, my research has illustrated a novel model for inter-plant volatile mediated communication in which GLVs produced by infected plants predispose neighboring plants for greater susceptibility. As illustrated in Figure 18, *C. graminicola* infected maize responds to fungal infection by increased expression of*LOX10* which in turn leads to GLV emission, specifically (Z)-3-Hexenal and (Z)-3-Hexenyl acetate. These GLVs are airborne signals to neighboring plants that induce JA, which activates proper defenses for insect attack but inappropriate for activation of defenses against hemibiotrophic pathogens.

Taken together, this study suggests an intriguing hypothesis with profound evolutionary consequences. Plants may have evolved capabilities to perceive GLVs as an eavesdropping

strategy to recognize the imminent threat by insects. Since herbivory by chewing insects is obviously more detrimental to plant fitness compared to relatively slow disease progression, it is possible that GLVs presence in PIPVs are "misinterpreted" as a signal to upregulate insect defenses instead of more appropriate SA-based defenses effective against hemibiotrophic pathogens such as *C. graminicola* and *C. heterostrophus*. Understanding the molecular underpinnings of pathogen spread leading to epidemics may provide novel environmentally conscientious approaches to control diseases.

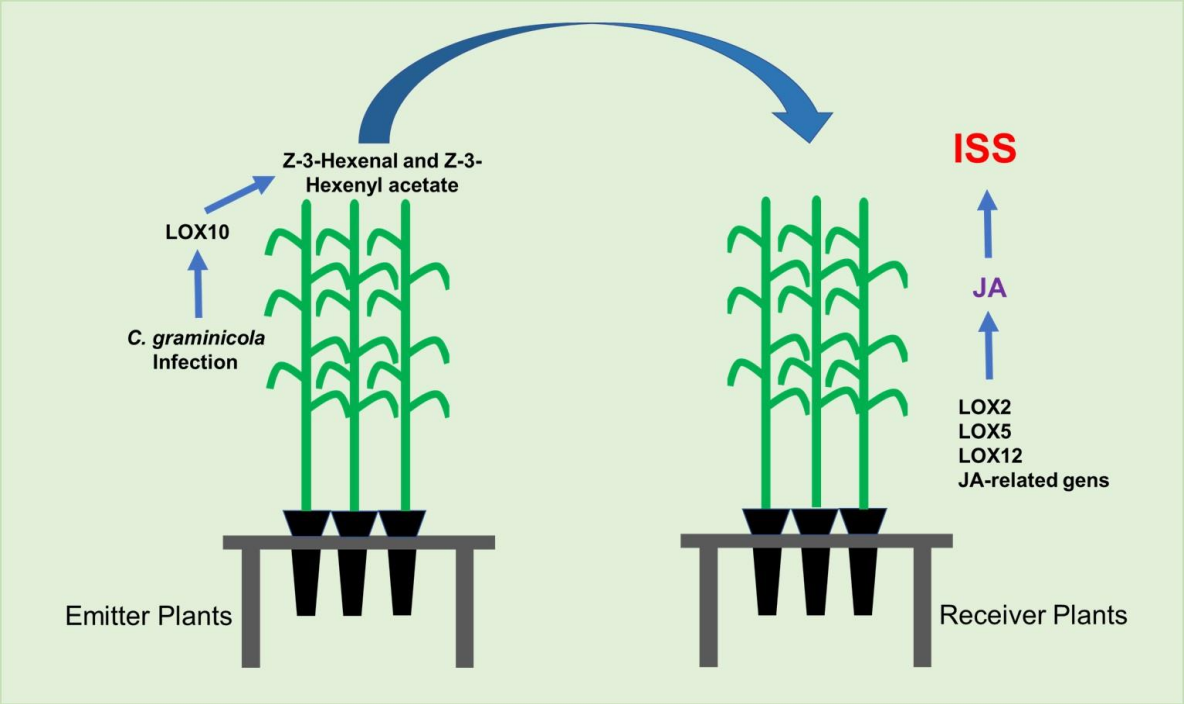

Figure 18: Model of PIPV induction of ISS. During a *C. graminicola* infection, LOX10 is induced and (Z)-3-Hexenal and (Z)-3-Hexenyl acetate are emitted. The GLVs signal an induction of JA-related genes, LOX5, and LOX12 which in turn results in the susceptibility hormone JA. JA leads to ISS when challenged with *C. graminicola*.

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