# THE FUNCTION OF THE LIPOXYGENASE ZMLOX10 IN MAIZE INTERACTIONS WITH INSECTS AND PATHOGENS

A Dissertation

by

SHAWN A. CHRISTENSEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2009

Major Subject: Genetics

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December 2009

Major Subject: Genetics

## ABSTRACT

The Function of the Lipoxygenase ZmLOX10 in Maize Interactions with Insects and Pathogens. (December 2009)

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Chair of Advisory Committee: Dr. Michael Kolomiets

Lipoxygenase (LOX)-derived oxylipins are known to play critical roles in defense against herbivores and pathogens. The objective of this study was to determine the biochemical, molecular and physiological roles of a specific maize lipoxygenase gene, *ZmLOX10*, with special emphasis on LOX10-derived oxylipins in plant-insect and plant-pathogen interactions. To achieve this goal, independent mutant alleles were generated and genetically advanced to create near-isogenic mutant and wild-type lines suitable for functional analysis. Here we provide genetic evidence that LOX10 is the sole LOX isoform in maize required for the biosynthesis of green leafy volatiles (GLV) in leaves and show that LOX10-mediated GLVs play a significant role in direct and indirect defense responses to insects through their regulation of jasmonic acid and volatile organic compound production. Contrary to the defensive role of LOX10 in plant-insect interactions, tests for susceptibility to fungal pathogens suggest that LOX10-mediated GLVs may contribute to the development of disease symptoms to the economically

important maize pathogens, *Aspergillus flavus* and *Colletotrichum graminicola*. Specifically, LOX10-derived GLVs may facilitate aflatoxin accumulation in response to *A. flavus* infection and may play a positive role in anthracnose leaf blight and stalk rot caused by *C. graminicola*. Collectively, our results suggest that metabolites derived from GLV-regulated pathways have a significant impact on molecular plant-herbivore and plant-pathogen interactions.

## ACKNOWLEDGEMENTS

Special thanks to my advisor, Dr. Mike Kolomiets, for giving me excellent council and guidance through my PhD program and for allowing me to freely explore my ideas and interests. Also, I would like to thank my committee members, Drs. James Starr, Hisashi Koiwa, and Brian Shaw, for their helpful advice and insightful reviews of my work. Many thanks to my collaborators, Jurgen Engelberth, Christian Nansen, Ivo Feussner, Conny Grobel, and James Tumlinson, for assistance in oxylipin, volatile, and biological analyses. Also, many thanks to my lab members/friends for helping along the way and for providing the means to enjoy the journey. Most deservingly, I would like to thank my wonderful wife, my children, and my family for their tireless support and willingness to endure to the end. Lastly, but always First, I would like to thank my Father in Heaven and His Son, Jesus Christ, who, quite literally, deserve the credit for any good that came from this work.

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# CHAPTER I

# INTRODUCTION

Recent discoveries associated with plant oxidized fatty acids, collectively called oxylipins, have had a riveting impact on plant physiology (Feussner and Wasternack, 2002; Howe and Jander, 2008) . The best understood plant oxylipins are those produced by the lipoxygenase (LOX) pathway. This pathway begins with the regio- and stereospecific dioxygenation of either linoleic (18:2) or α-linolenic acid (18:3) to form 9- or 13-hydroperoxides (HPOTE), depending on the site of molecular oxygen addition. These 9- and 13-HPOTEs are subsequently used as substrate for the seven branches of the LOX pathway including the peroxygenase, divinyl ether synthase, reductase, epoxy alcohol synthase, hydroperoxide lyase (HPL), allene oxide synthase (AOS), and LOX reactions (Feussner and Wasternack, 2002). In conjunction with LOX enzymatic activity that yields pure oxylipin enantiomers, chemical lipid peroxidation can yield oxylipin products similar to those derived from enzymatic reactions or can also produce different positional isomers than those synthesized by LOXs. For example, the formation of reactive oxygen species can lead to lipid peroxidation at the C-9 and C-13 carbon positions to form racemic mixtures of oxylipins similar to LOX derived metabolites. Moreover, autooxidation can also form unique lipid peroxides at the C-10 and C-12 positions (Andreou et al., 2009; Mosblech et al., 2009).

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This dissertation follows the style of *Plant Cell*.

To date, LOX-derived oxylipins have been implicated in playing important roles in a variety of developmental processes and defense responses to insects and pathogens. For example, the product of the AOS branch of the LOX pathway, jasmonic acid (JA), plays a developmental role in root growth, seed germination, tuber and trichome formation, senescence, and flower development. Furthermore, JA plays a significant role in direct and indirect defense responses to herbivory by regulating the expression of protease inhibitors and the release of volatile organic compounds (Howe and Jander, 2008). Although less understood, the products of the HPL branch, GLVs, are also known to induce defense related genes (Bate and Rothstein, 1998), have direct anti-fungal and anti-bacterial activities (Shiojiri et al., 2006; Prost et al., 2005), and act as volatile signals to prime neighboring plants in preparation for insect attack (Engelberth et al., 2004). While the role of these and other oxylipins have been studied in dicotyledonous species, their biosynthesis and function in maize and other monocotyledonous species remains obscure. Therefore, the major goal of this PhD study was to broaden this knowledge base and identify the role of a subset of the plant oxylipins, namely those derived from the 13-LOX enzymatic reaction in maize. The information gleaned from this research is expected to improve our understanding of plant defense mechanisms and may help design novel strategies to enhance resistance to harmful herbivores and pathogens.

The specific objective of this study was to determine the biochemical, molecular and physiological roles of a maize LOX gene, *ZmLOX10*, with special

emphasis on LOX10-derived oxylipins in plant-insect and plant-pathogen interactions. An important basis for this objective is the previously published data and my own preliminary results that helped form my central hypothesis that LOX10 is responsible for feeding substrate to the HPL branch of the LOX pathway for GLV biosynthesis. I further hypothesized that the deficiency of GLVs in *lox10* mutants would result in decreased resistance to herbivores and pathogens. To test the central hypothesis *lox10* mutants and near-isogenic wild-types were used to pursue two specific objectives:

1) determine the biochemical and physiological roles of LOX10 in response to mechanical wounding and insect damage;

2) characterize the function of LOX10 in resistance to the economically important maize pathogens *Colletotrichum graminicola* and *Aspergillus flavus*.

The knowledge gained from this study is expected to enable scientists, plant breeders, and farmers alike to utilize LOX10 to enhance herbivore- and pathogen-related defense responses in order to increase crop productivity and minimize the environmentally hazardous and costly nature of current pest control methods.

# CHAPTER II

# LOX10 IS RESPONSIBLE FOR GLV BIOSYNTHESIS:

# ROLE IN REGULATION OF JA, VOC, AND HERBIVORE DEFENSE

## Introduction

As immobile entities in nature, plants are constantly forced to stand their ground and survive the continuous attacks by harmful insect invaders[1]. In response to these stresses, plant tissue undergoes a reprogramming of genetic and metabolic processes to facilitate direct and indirect defense responses (Maffei et al., 2006). As direct countermeasures, toxins and defensive proteins are imparted to repel insects and frustrate digestibility. Indirect defense includes the release of volatile organic compounds (VOCs) that are deployed to attract insect predators and parasitoids, enabling them to hone in on their prey (Howe and Jander, 2008). While the detailed molecular and biochemical regulation of these responses is still obscure, it is widely known that much of the essential components involve terpenes and metabolic products from the fatty acid biosynthetic and signaling pathways including metabolites derived from lipoxygenases (LOX)(Pare and Tumlinson 1997, 1999).

[1] This chapter is in preparation for journal submission. Contributing researchers that will be listed as authors on the publication are: Shawn Christensen, Andriy Nemchenko, Jurgen Engelberth, Jim Tumlinson, Liz Bozak, Christian Nansen, Ivo Feussner and Mike Kolomiets. All the experiments were carried out by myself with the exception of volatile organic compound collections (assisted by Jim Tumlinson, Jurgen Engelberth, and Liz Bozak) and beet armyworm assays (assisted by Christian Nansen).

LOX-derived metabolites, called oxylipins, are well known for their roles in biotic and abiotic stress responses. Initiation of the LOX pathway begins when polyunsaturated fatty acids [linoleic (18:2) and linolenic (18:3) acid] are cleaved from cell membranes by diverse lipases and dioxygenated by either 9- or 13-LOXs. Subsequent products from the 9- and 13-LOX reactions include either 18:2 derived (9S)-hydroperoxyoctadecadienoic acid (9-HPODE) and (13S)-hydroperoxyoctadecadienoic acid (13-HPODE) or the 18:3 dervaitives (9S)-hydroperoxyoctadecatrienoic acid (9-HPOTE) and (13S)-hydroperoxyoctadecatrienoic acid (13-HPOTE). These hydroperoxides act as substrate for seven alternative branches of the LOX pathway, namely peroxygenases, divinyl ether synthases, reductases, epoxy alcohol synthases, hydroperoxide lyases (HPL), allene oxide synthases (AOS), and subsequent LOX reactions for the assembly of numerous oxylipins (Feussner and Wasternack 2002). While there is yet much to unveil in terms of the physiological roles of plant oxylipins, current literature on wound and herbivory responses focus principally on the AOS and HPL pathway branches, responsible for the biosynthesis of jasmonic acid (JA) and green leafy volatiles (GLVs), respectively.

The synthesis of JA begins with LOX derived 13-HPOTE, which is catalyzed into epoxides by AOS, transformed into a 5-carbon ring via allene oxide cyclase, reduced by 12-oxo phytodienoic acid reductase (OPR), and truncated by three beta-oxidation steps to form (+)-7-iso-JA (Wasternack, 2007). JA and/or its metabolites maintain a central role in herbivore induced defense responses. This

is evident by the numerous studies conducted where JA is shown to have a regulatory influence on tritrophic interactions, resistance to phloem feeders, trichome-centered defenses, priming of indirect and direct defenses, pathogen resistance, and systemic defense signaling (Howe and Jander, 2008).

Similar to JA, the formation of the HPL derived constituents begins with the cleavage of 13-HPOTE to form cis-3-hexenal, which is further enzymatically processed to produce other C6-compounds including cis-3-hexenol and cis-3-hexenyl acetate (Ble´e, 2002; D'Auria et al., 2002; Matsui, 2006). GLVs possess both anti-bacterial and anti-fungal properties, although some accounts show them to be fungal susceptibility factors when the fungus is exposed to lower levels of C-6 volatiles (Prost et al., 2005; Matsui, 2006). GLVs are known for their roles in herbivore defense. While some studies show GLVs to be insect repellants (i.e. anti-HPL mutants in potato are more susceptible to aphids; Vancanneyt et al., 2001), GLVs can also increase a plant's herbivore appeal (i.e. anti-HPL mutants in tobacco are less attractive to *Manduca sexta*; Halitschke et al., 2004). GLVs are recognized as strong signaling molecules that regulate plant-plant communication after insect elicitation (Arimura et al., 2000; Farag and Pare, 2002; Engelberth et al., 2004) and induce the expression of defensive genes (Bate and Rothstein, 1998). The exposure of maize plants to exogenous GLVs induces VOC and JA production, and more importantly, enhances the JA response to herbivore attack (Kessler and Baldwin, 2001; Engelberth et al., 2004). These microbial and herbivore related examples show the large number of organisms that interact with

GLVs and demonstrate the varied effects GLVs can have, based on the co-evolution of the plant and species involved.

The specific signaling mechanisms behind JA- and GLV-mediated defense responses to wounding and herbivory are poorly understood, especially in maize. This may, in part, be a result of various technical challenges associated with LOXs being encoded by large multigene families, expressed in different tissues, producing different compounds, and regulated differentially by biotic and abiotic stresses. These facts alone are suggestive of their specialization in producing different oxylipins under different treatments and it is, therefore, likely that specific isozyme forms are responsible for providing substrate to specific pathway branches. For example, of multiple LOX isoforms found in tomato and potato, disruption of LOX-H1 and tomato TomLOXC show reduced levels of GLVs after tissue damage, but no changes in JA production (Leon et al., 2002; Chen et al., 2004). Alternatively, *Arabidopsis* AtLOX2 and tobacco, NaLOX3 affect the JA biosynthetic pathway, yet forgo any function in GLV biosynthesis. Collectively, these studies show that diverse LOXs have specific functions, which may be a result of the intracellular spatial separation that exists between the different LOX pathways. Furthermore, these studies show that until now, the HPL and AOS branches of the LOX pathway have not been interdependent.

Here we report on the characterization of the two 13-LOXs that appear to be specifically responsible for either GLV or JA biosynthesis in maize and show that signaling cross-talk exists between the HPL and AOS branches of the LOX

pathway. Specifically, we provide evidence that mutations in the 13-LOX isoform, *ZmLOX10*, completely eliminate GLV biosynthesis in maize. Moreover, we show that *ZmLOX8* [also known as tasselseed1 (ts1)] appears to be responsible for wound-induced JA production, and demonstrate that both JA and VOC production in maize is GLV-dependent. The reduction of GLVs in *lox10* mutants leads to compromised resistance to beet armyworm (BAW), which confirms the defensive role of this gene.

## Results

### Clustering of putative GLV- and JA-producing LOXs in maize

The major objective of this study is to identify maize 13-LOXs involved in wound- and herbivore-induced GLV and JA biosynthesis. To begin our study, we phylogenetically compared the previously identified maize 13-LOXs (Nemchenko et al., 2006; Gao et al., 2007; Kolomiets et al., unpublished data) with 13-LOXs from dicotyledonous species that had previously been characterized to have roles in either JA (NaLOX3, Halitsche and Baldwin, 2003; StLOXH-3, Royo et al., 1999; TomLOXD, Heitz et al., 1997) or GLV (TomLOXC, Chen et al., 2004; StLOXH-1, Leon et al., 2002) production. The dendrogram revealed two discrete clusters, one that included LOX genes associated with GLV biosynthesis and the other that included LOXs associated with JA production (Figure 1). ZmLOX10 and

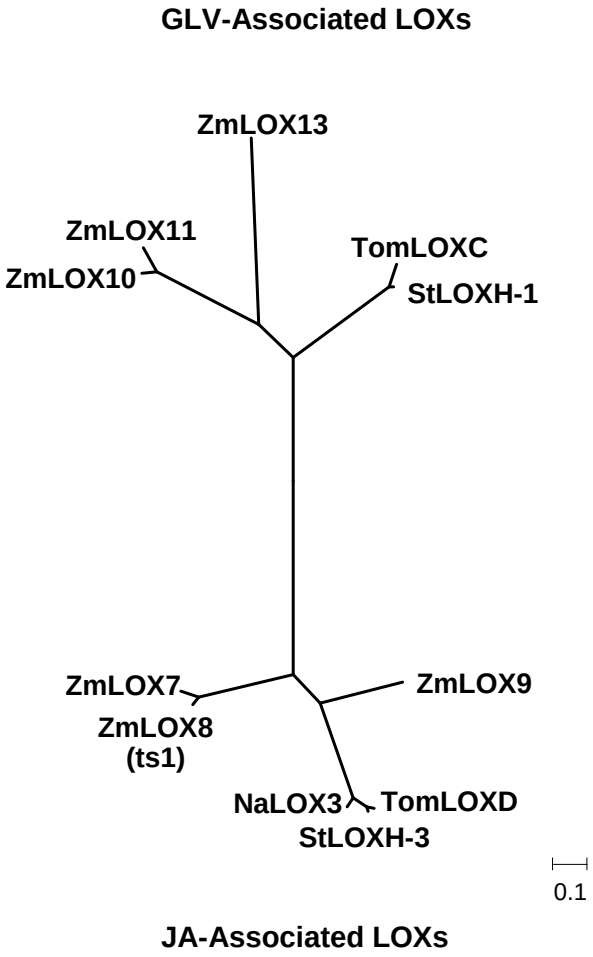


Figure 1. Phylogeny of GLV- and JA-associated LOXs from different plant species.

A maximum likelihood tree was built by aligning protein sequence from maize 13-LOXs and other GLV- (TomLOXC and StLOXH-1) or JA- (TomLOXD, StLOXH-3, and NaLOX3) relevant 13-LOXs in tomato, potato, and tobacco using Clustal W software (Guindon and Gascuel 2003). Phyologeny was reconstructed using the LG substitution model implemented in PHYLM 3.0 software. The two clustered lineages support the biochemical functions between GLV- and JA-associated LOXs.

ZmLOX11 showed a high level of homology between themselves (>90%; Nemchenko et al., 2006), and grouped with ZmLOX13 and the other GLV-producing LOXs. ZmLOX7 and ZmLOX8 were likewise highly similar at the amino acid level and grouped with ZmLOX9 and the other JA-associated LOX genes. Because of its relatedness to other GLV-producing LOXs, we hypothesized that LOX10 is likely involved in the biosynthesis of GLVs (Nemchenko et al., 2006). Because LOX7, 8 and 9 cluster together with other JA-producing dicot LOXs, we hypothesized that these isoforms feed the hydroperoxy linolenic acid into the AOS branch of JA biosynthesis.

Generation of transposon-insertional *lox10* mutants

To identify mutant alleles of *ZmLOX10 and ZmLOX11*, we utilized the TUSC reverse genetics resource at Pioneer/DuPont. Unfortunately, no mutants were detected for *ZmLOX11*; however, three individual alleles were identified at the *ZmLOX10* locus (*lox10-1*, *lox10-2*, and *lox10-3*). Sequencing of the regions flanking insertion sites showed that the *lox10-1* allele harbored a *Mu*-element in the first intron while *lox10-2* and *lox10-3* were exonic alleles with the Mu-element inserted in exon III (Figure 2A). Because *ZmLOX10* is induced to its greatest levels 6-12 h following mechanical wounding (Nemchenko et al., 2006), transcript accumulation was measured 8 h post wounding in *lox10-2* and *lox10-3* mutant leaves. As reported previously, WT plants showed a clear induction of Zm*LOX10*

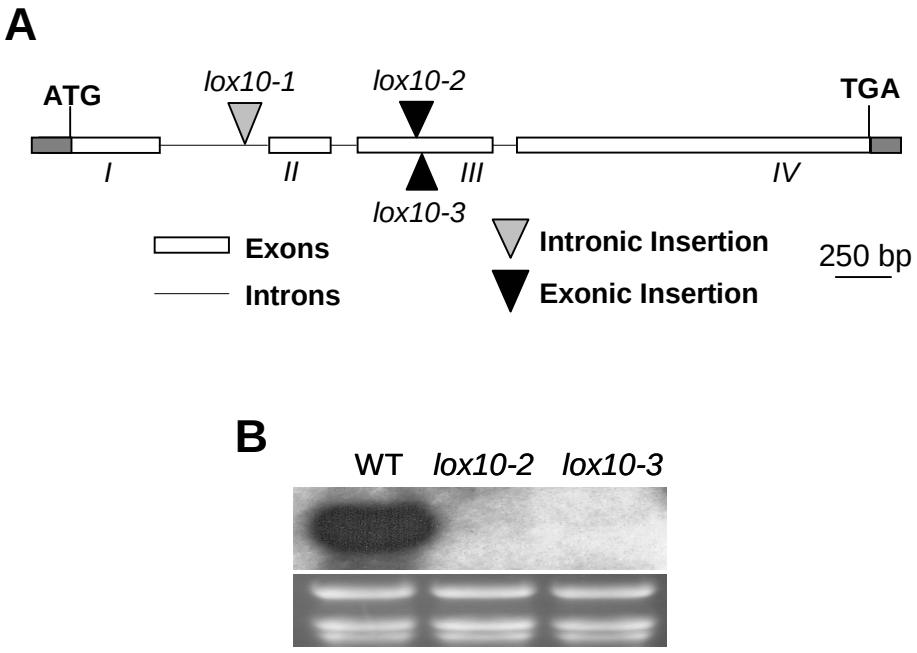


Figure 2. *Mu*-insertions in the *lox10-2* and *lox10-3* mutant alleles resulted in suppression of *ZmLOX10* gene expression suggesting that they are null transcript alleles.

(A) Schematic diagram of *Mu* insertion sites in the *ZmLOX10* gene. *lox10-2* and *lox10-3* are exonic insertions.
(B) Northern blot analysis of *ZmLOX10* transcript levels in WT and *lox10-2* and *lox10-3* mutants 8 h post-wounding. Twelve micrograms of total RNA was transferred to a nylon membrane and hybridized with the *ZmLOX10* gene-specific probe. Equivalent loading of RNA was visualized by ethidium bromide staining and UV transillumination.

transcripts, whereas no detectable hybridization signal could be observed for both *lox10-2* and *lox10-3* alleles (Figure 2B). These data indicate that *ZmLOX10* transcripts do not accumulate in either the *lox10-2* or *lox10-3* mutants, providing evidence that they may be null alleles. In working with mutants derived from mutator transposition, the possibility exists that genes integrated into the mutant lines by linkage drag could be responsible for phenotypic differences between WT and mutant plants. However, because both *lox10-2* or *lox10-3* mutant alleles came from independent *Mu*-active lines, the likelihood of linked genes being responsible for the same phenotype in both mutant alleles greatly diminishes.

*lox10* mutants are GLV deficient upon wounding and caterpillar feeding

The clustering of LOX10 with two other GLV-producing isoforms prompted us to test our hypothesis that LOX10 feeds substrate to the HPL pathway. Since GLVs are emitted rapidly upon mechanical wounding, WT, *lox10-2* and *lox10-3* mutant seedlings were wounded by hemostat and head-space volatiles were collected and measured 10 min post treatment. The chromatogram (Figure 3A) shows that the GLVs Z-3-hexenal, Z-3-hexenol, and Z-3-hexenyl acetate were not detected in wounded *lox10-2* and *lox10-3* mutant leaves, whereas WT plants emitted detectable levels of all three volatiles. GLV emission was further measured in

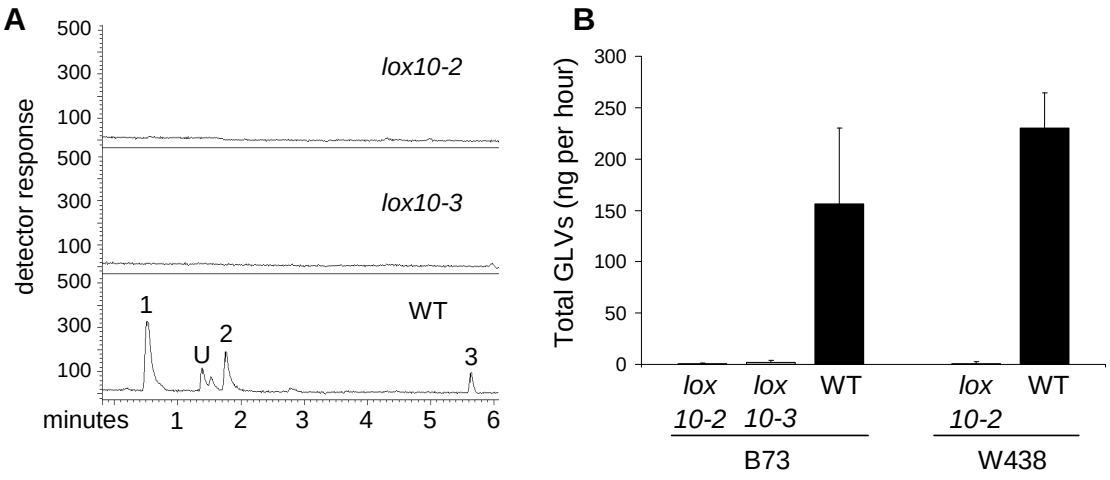


Figure 3. ZmLOX10 is responsible for GLVs in leaves of wounded and BAW infested maize plants.

(A) Gas chromatogram (GC/MS) showing volatile emissions from mechanically-wounded leaves of WT and *lox10* mutant plants. Volatiles are labeled numerically as follows: (1) Z-3-hexenal, (2) Z-3-hexenol, and (3) hexenyl acetate. U = Unknown compound.
(B) Quantification of total GLV emissions in *lox10-2* and *lox10-3* mutant and wild type seedlings 2-4 h post-infestation with beat armyworm (*Spodoptera exigua*). Near-isogenic mutant and wild type lines were created in the B73 and W438 genetic background. Measurement of selected volatile emissions from maize seedlings presented as total GLVs (B73: Z-3-hexenal, E-2-hexenal, Z-3-hexen-1-ol, and Z-3-hexenyl acetate; W438: Z-3-hexenal, E-2-hexenal, Z-3-hexen-1-ol, Z-3-hexenyl isobutyrate, and Z-3-hexenyl acetate). Data represented as means ± standard deviation (SD).

response to feeding of seedlings by beet army worms (BAW) between 2-4 h post infestation. Normal levels of total GLVs (combined values for Z-3-hexenal, Z-3-hexenol, Z-3-hexenyl acetate and E-2-hexenal) were emitted by infested WT plants (156 ng/h), whereas, *lox10-2* and *lox10-3* mutant emissions were comparable to background levels (0.4 and 1.7 ng/hr, respectively; Figure 3B). These results suggest that LOX10 is the sole 13-LOX isoform responsible for catalyzing 13S-HPOTE for the HPL pathway branch for GLV production in maize leaves in response to wounding or herbivory by BAW.

*lox10* mutants produce lower wound-induced levels of JA and OPDA

Because LOX10 is predominantly a 13-LOX (Nemchenko et al., 2006), the biosynthesis of jasmonates was another potential function of this isoform. To test this hypothesis, OPDA and JA levels were measured in young leaves upon mechanical wounding using both *lox10-2* and *lox10-3* mutants alleles and near-isogenic WT in the B73 genetic background. There was no significant difference in the levels of JA for non-wounded (0 h time point) WT and *lox10-2* and *lox10-3* mutants ($P \geq 0.25$). However, 2 h post wounding WT levels showed a 4-fold increase in JA, compared to *lox10-2* and *lox10-3* mutants ($P \leq 0.001$; Figure 4A). By 12 h post-treatment, WT JA levels were still 2-fold higher than *lox10-2* and *lox10-3* mutants ($P \leq 0.05$). Similarly, accumulation of cis-OPDA, the natural JA precursor, was not different between mutants and WT at 0 h ($P \geq 0.20$), but was

significantly higher in the WT 2 h after wounding ($P \leq 0.01$; Figure 4B). Twelve hours post wounding, OPDA levels in WT were still significantly higher than *lox10-2* and *lox10-3* mutants ($P \leq 0.01$). Taken together, these data indicate that *ZmLOX10* is involved in the wound-induced regulation of biosynthesis of both OPDA and JA but not in the production of basal levels of these octadecanoids.

*ZmLOX8* is responsible for wound-induced JA in maize

To determine if another maize 13-LOX is required for wound-induced JA production, we wounded seedlings and measured transcript accumulation of the maize 13-LOXs (*ZmLOX7*, *ZmLOX8*, and *ZmLOX9*) that clustered with the previously characterized JA-associated LOX genes from other plant species (Figure 1). Of the three candidate genes tested, we found that *ZmLOX8*, a gene previously characterized to have a function in JA-mediated tassel development (also known as *tasselseed1*; Acosta et al., 2009), was the only 13-LOX isoform strongly induced by wounding (Figure 5A and data not shown). Using the publicly available *tasselseed1-ref* (*ts1-ref*) allele, a knockout mutant of the *LOX8* locus, we measured wound-induced JA levels in homozygous WT and mutant seedlings from the F2 segregating family. Figure 5A shows that *lox8/ts1-ref* mutants (here after denoted as *lox8*) produced 51% less JA than WT in response to wounding

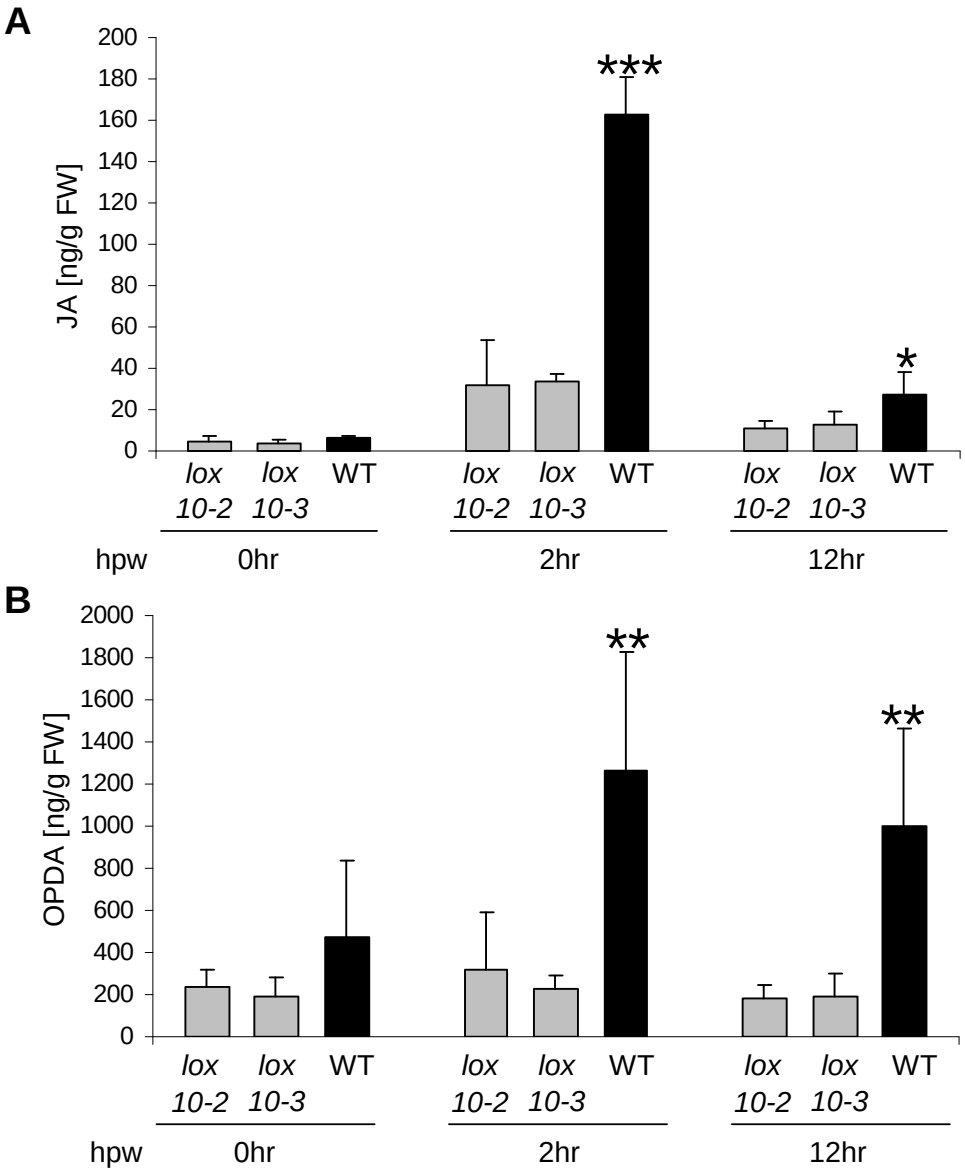


Figure 4. Mutant *lox10-2* and *lox10-3* alleles produce reduced levels of OPDA and JA in response to mechanical wounding. Quantification of OPDA (A) and JA (B) levels (mean ± SD) in maize seedlings 0, 2, and 12 h post wounding by hemostat (n = 4). Asterisks specify significant differences between WT and mutants under comparable treatments (ANOVA, Tukey pairwise comparison; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

($P<0.05$). These results suggest that LOX8 may be the maize isoform responsible for feeding substrate to the wound-induced JA biosynthesis pathway.

LOX10-mediated GLVs are required for normal expression levels of JA biosynthesis genes

To elucidate the potential mechanism of LOX10 involvement in the regulation of wound-induced JA biosynthesis, transcript accumulation of other putative JA-producing enzymes were measured in wounded leaves over a time course spanning 8 h. In addition to the previously described LOX8, the other characterized putative JA producing enzymes in the maize genome are OPR7 and/or OPR8 (Zhang et al., 2005). These two genes are the only OPRII genes that phylogenetically cluster with the *Arabidopsis* JA producing enzyme *AtOPR3* (Zhang et al., 2005; Schaller et al., 2000). Northern blotting analysis showed that WT seedling transcript levels of *ZmLOX8* increased strongly as early as 0.5 h after wounding and then returned to basal levels 4 h post treatment (Figure 5B). Similar differences between WT and *lox10-2* and *lox10*-3 plants were seen in *ZmOPR7/8* gene expression with the maximal transcript accumulation occurring at 2 h post wounding in the WT. Combined, these results suggest that LOX10-mediated metabolism is required for normal wound-induced expression of JA-producing *ZmLOX8* and putative JA-producing *ZmOPR7/8* and normal levels of JA biosynthesis in response to mechanical damage.

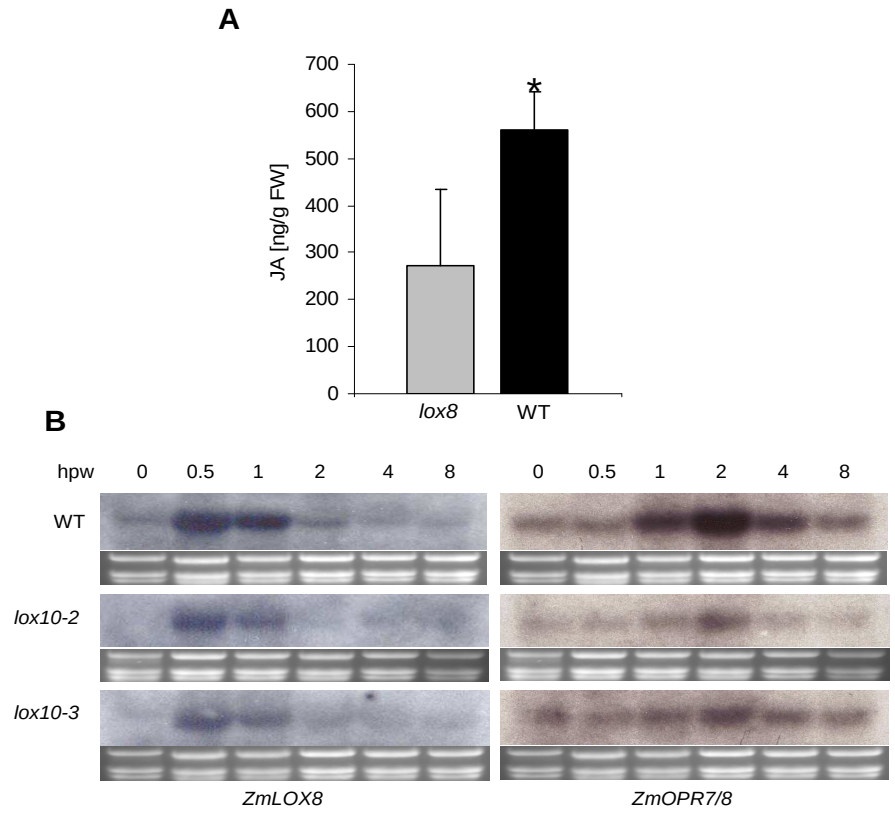


Figure 5. Disruption of *ZmLOX10* resulted in reduced transcript levels of *ZmLOX8* and *ZmOPR7/8*, the genes involved in jasmonic acid biosynthesis.

(A) *ZmLOX8 (tassel seed 1*) mutants produce lower levels of jasmonic acid in response to mechanical wounding of leaves. JA was measured by liquid chromatography-mass spectrometry in wounded leaves of seedlings segregating for the *ts1-ref* allele 2 h post treatment. *lox8*, homozygotes for the ZmLOX8 gene; WT, homozygous wild types. The genotypes of the mutants were determined by PCR using *LOX8* gene-specific probes as described in Acosta et al. (2009); (mean ± SD; ANOVA; * $P \leq 0.05$).
(B) *ZmLOX8* and *ZmOPR7/8* transcript accumulation in response to wounding of *lox10-2* and *lox10-3* mutant and wild-type seedlings. The second leaf from maize seedlings were mechanically wounded with a hemostat and collected at 0, 0.5, 1, 2, 4, and 8 h post wounding. Total RNA was transferred to a nylon membrane and hybridized with either a *ZmLOX8* or *ZmOPR7/8* probe. Equal loading of RNA was visualized by ethidium bromide staining and UV transillumination.

LOX10-mediated GLVs regulate VOC emissions

To test if LOX10-mediated GLVs would alter VOC emission during caterpillar feeding, WT and *lox10-2* and *lox10-3* mutant seedlings were infested with 3rd instar larvae BAW and GLVs, monoterpenes, homoterpenes, and sesquiterpenes were collected over a 2 h period. As expected, total GLVs (Z-3-hexenal, E-2-hexenal, Z-3-hexen-1-ol, and Z-3-hexenyl acetate) were virtually absent in *lox10-2* and *lox10-3* mutants as compared to the WT plants ($P < 0.001$; Figure 3B). Emissions of monoterpenes (α-phellandrene, 3-carene, alpha terpinene, limonene, ocimene, geranyl acetate, caryophyllene, β -pinene, and β-myrcene) and sesquiterpenes (copaene, cedrene, caryophyllene, bergamotene, E-beta-farnesene, and bicyclosesquiphellandrene) in *lox10-2* and *lox10-3* mutants were 2-fold less than emissions from WT plants ($P \leq 0.03$; $P \leq 0.04$, respectively; Figure 6A;). Moreover, levels of *lox10* mutant homoterpene (4,8-dimethyl-1,3,7-nonatriene and 4,8,12-trimethyl-1,3,7,11-tridecatetriene) emissions were 3-fold less than those emitted by WT ($P \leq 0.02$). In addition to B73, we tested WT and mutants in the W438 genetic background, which appeared to produce greater levels of GLVs (Figure 3B). To assess the impact of GLVs on the production of VOCs, we measured head space emission levels in W438 and *lox10-2* mutants during BAW feeding over a 7.5 h time course. Here, even greater differences in VOCs were observed between WT and *lox10-2* mutant plants. As expected, GLVs

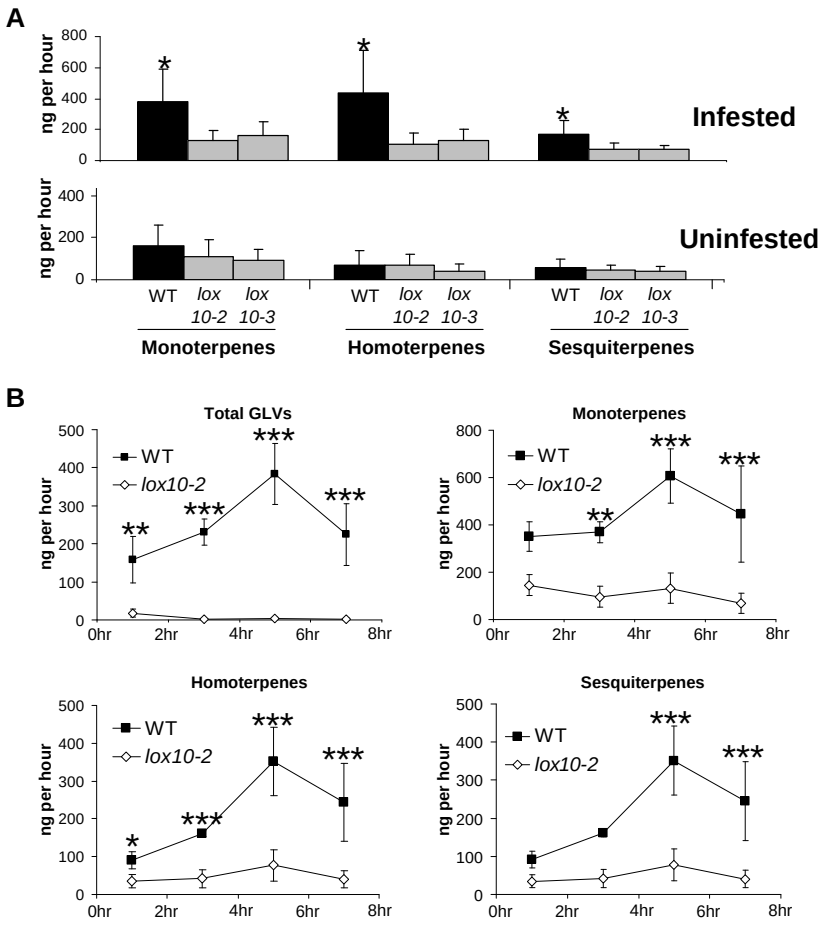


Figure 6. Volatile organic compound (VOC) emissions from beat armyworm infested maize plants.

(A) VOC emissions (mean ± SD; ng per hour) in beat armyworm (*Spodoptera exigua*) infested wild type and *lox10-2* and *lox10-3* mutant plants in the B73 genetic background. Bars represent mono-, homo-, and sesquiterpene emissions during a 2 h collection period.
(B) Emanation of VOCs (mean ± SD; ng per hour) from wild type and *lox10-2* mutant infested maize plants in the W438 genetic background in a 7.5 h time course. Significant differences in GLVs, monoterpenes, homoterpenes, and sesquiterpenes are indicated by asterisks (ANOVA, Tukey pairwise comparison; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

were virtually absent in *lox10-2* mutants at all time points. Monoterpenes and homoterpenes both showed strong induction 2-4 h post infestation demonstrating significant differences between WT and mutant ($P \leq 0.0076$; $P \leq 0.001$, respectively). Moreover, WT levels for all three classes of volatile terpenes were significantly higher than the mutant at 4-5.5 h post infestation (monoterpenes, $P \leq 0.001$; homoterpenes, $P \leq 0.001$; sesquiterpenes $P \leq 0.001$; Figure 6B). The results from these two experiments in two genetic backgrounds strongly advocate that *ZmLOX10* has an important function in the regulation of VOC production in response to insect herbivory.

*lox10-2* and *lox10-3* mutants are more susceptible to beat armyworms

The regulatory effect of LOX10 on herbivore defense-related compounds prompted us to measure its biological relevance to insect attack. In order to test this, we infested WT and *lox10-2* and *lox10-3* mutant plants with 2$^{nd}$ instar BAW and monitored caterpillar performance by measuring weight gain 6 d after feeding. Beet armyworm that fed on WT plants gained significantly less weight than both *lox10-2* ($P = 0.0265$) and *lox10-3* ($P = 0.0407$) mutants (Figure 7A). In addition,

we compared fresh weight of the control uninfested WT and *lox10-2* and *lox10-3* mutant plants to their counterpart infested plants 6 d after feeding. Weight of the WT infested plant was not significantly different from that of the control non-infested plant after BAW feeding (Figure 7B; $P = 0.5456$). On the contrary, both infested *lox10-2* and *lox10-3* mutant plants weighed significantly less than their non-infested equivalents ($P = 0.03$ and $P = 0.05$, respectively), indicating that the BAW consumed more plant tissue when feeding on mutant plants as apposed to WT. To measure BAW eating preference, we placed BAW in pots with WT, *lox10-2*, and *lox10-3* plants and then compared initial and post-feeding plant weights. Figure 7C shows that both *lox10-2* and *lox10-3* mutant plants decreased in weight over the 6 d feeding period, whereas WT plants showed increased weight gain. Collectively, these results suggest that *ZmLOX10* has a direct biological role in resistance to BAW feeding.

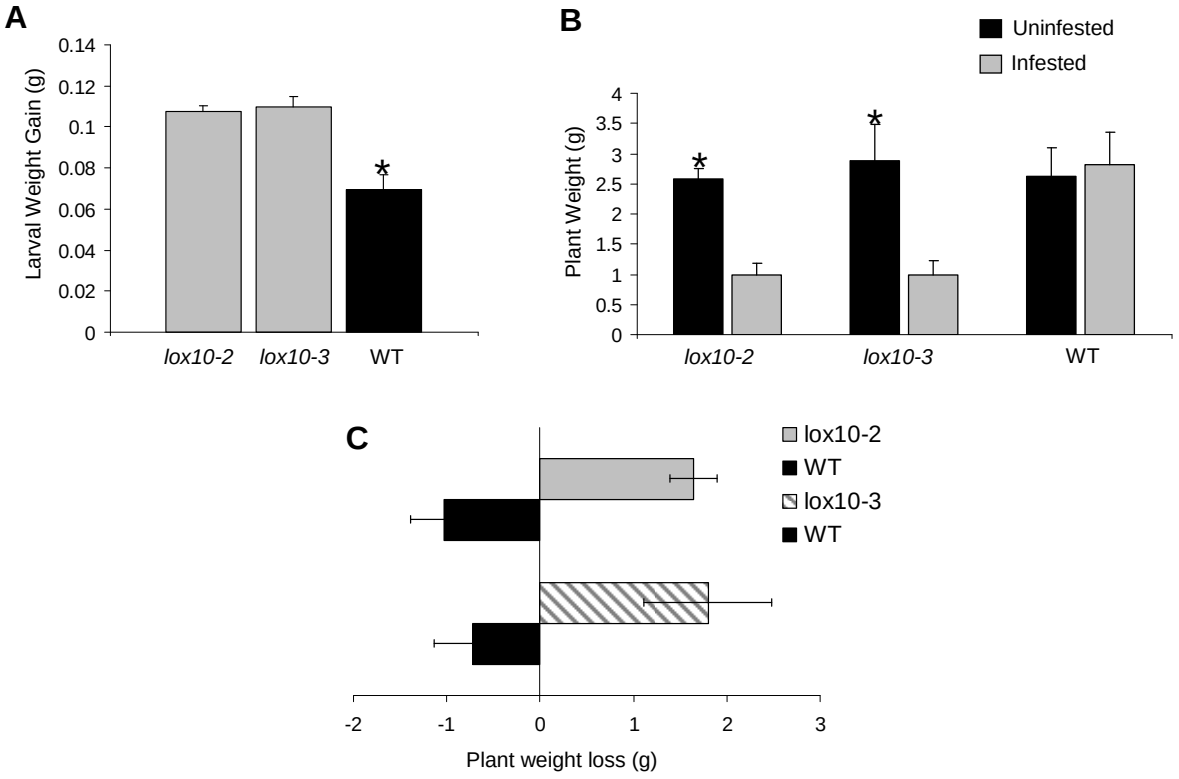


Figure 7. Herbivore performance and preference bioassays with beet armyworm (*Spodoptera exigua*).

(A) and (B) Performance bioassays. Separate pots of WT, *lox10-2*, or *lox10-3* maize plants were infested with a single 2nd instar *Spodoptera exigua* larva. Six days post-infestation, plants and larvae were collected and weighed. (A) Larval weight gain 6 d post infestation. (B) Infested plants (grey) compared to uninfested (black) controls.
(C) Preference bioassays. Pots containing WT, *lox10-2,* and *lox10-3* were infested with a single *Spodoptera exigua* larva. Plant weight was measured 6 d post infestation. Graph represents plant weight loss as determined by comparison of infested plants with their respective uninfested controls (mean ± SD; Kruskal-Wallis Test; * $P \leq 0.05$).

## Discussion

Plants have evolved an innate ability to thwart insect attack through the regulation of direct and indirect defense responses. The underlying mechanisms of insect resistance signaling is still fragmentary, yet emerging evidence suggests that the major strategy of maize and other plants involves diverse lipid oxidation products that serve as defense signals, including those derived from the HPL and AOS branches of the lipoxygenase (LOX) pathway. To date, HPL derivatives, namely GLVs, have been shown to play a role in the priming of JA- and VOC- mediated defense responses in neighboring plants, but their involvement in direct defense responses for the plant under attack has been underestimated. This study challenges the assumption that GLVs play a minimal role in direct defense response and further bridges the mechanistic gap between herbivore damage and indirect defense. Specifically, we provide genetic evidence that the JA- and VOC-mediated defense responses required for resistance to insect herbivory in maize are GLV-dependent.

One of the principle findings of this study is that among the 13 individual LOX genes identified in the maize genome (Kolomiets et al., unpublished), LOX10 is the sole isoform responsible for generating linolenic acid hydroperoxide (13-HPOTE) substrate for the GLV biosynthesis pathway in leaves. Evidence implicating LOX10s involvement in providing substrate for the HPL branch of the 13-LOX pathway was demonstrated in both wounding and herbivore related

treatments where *lox10-2* and *lox10*-3 mutants were virtually GLV-deficient. This surprisingly occurs in the presence of five other functional 13-LOXs including *ZmLOX11*, which is a segmentally duplicated gene that shares >90% identity at the amino acid level. Nemchenko et al., (2006) showed that *ZmLOX10* and *ZmLOX11* are differentially expressed under diverse treatments and in an organ-specific manner, which suggests that the biochemical and perhaps physiological functions of these paralogs diverged since their duplication event, possibly as a result of minimal selection pressure on *ZmLOX11* in the presence of a functional *ZmLOX10*. The fact that *ZmLOX10* is uniquely responsible for GLV biosynthesis in leaves in the presence of *ZmLOX11* and the four other 13-LOXs underscores the functional significance of the differential transcriptional regulation, subcellular localization and tissue specific spatial organization that exists among different LOX isoforms in planta. This being the case, *ZmLOX11* may have a function in GLV production but is limited to specific organs other than leaves (i.e. silks; Nemchenko et al., 2006)

LOX8 likewise demonstrates the importance of the biochemical specialization of LOX enzymes in maize. Although it clusters with two other putative JA producing isoforms (ZmLOX7 and ZmLOX9; Figure 1), its disruption singularly is sufficient to significantly reduce wound-induced production of JA. This suggests that LOX8 is not only responsible for the JA production required for tassel development (Acosta et al., 2009), but it plays an important role in the biosynthesis of JA for wound-induced responses in leaves. This finding

underscores the non-redundant role that *ZmLOX8* plays in tassel development (Acosta et al., 2009) and wound-regulated JA (Figure 5). This also gives rise to the possibility that its paralogs, *ZmLOX7* and *ZmLOX9*, may functionally produce JA in other organs and/or under different stresses or developmental conditions.

While ZmLOX8 clusters with and has similar biochemical function to JA-producing LOXs from other plant species, it appears to be unique in its method of induction. The reduction of transcript levels of both *ZmLOX8* and *ZmOPR7/8* in *lox10* mutants confirms the dependence of these JA biosynthetic genes on LOX10-mediated GLVs for normal wound-induced levels of transcript and JA (Figures 4 and 5). While the regulation of JA biosynthesis by exogenous GLV application has already been shown (Engelberth et al., 2004, 2007), we provide the first genetic evidence and putative mechanism that within-plant GLVs have signaling properties that regulate JA production.

This cross talk between the HPL and AOS branches of the LOX pathway in maize is different from what has been previously reported. To the best of our knowledge, only two other genetic studies have addressed specific interaction of these two pathways (Halitschke et al., 2004; Chehab et al., 2008). In both reports, there was an inverse relationship between GLVs and JA, which appeared to be a result of substrate competition. In maize, it appears that there is no substrate competition for the HPL and AOS branches of the LOX pathway, which suggests that they may be spatially separate from one another, much like those described by Froehlich et al. (2001) in tomato.

That GLVs are regulated by LOX10 and have been previously shown to induce JA (Engelberth et al., 2004, 2007), supports the argument that LOX10-mediated GLVs are the signaling molecules that regulate wound-induced JA biosynthesis. Additionally, LOX10 may provide substrate directly to the JA biosynthesis pathway. Future analyses with maize mutants specific to the GLV biosynthesis pathway (i.e. *hpl*) will help to elucidate the definitive mechanism for LOX10 involvement in JA biosynthesis.

Perhaps the most intriguing discovery of this study is the reduction of VOCs in *lox10* mutants. Mono-, homo-, and sesquiterpene levels were reduced during herbivore feeding for assays ran in both the B73 and W438 genetic backgrounds. While several sources have reported the elicitation of VOCs following exogenous GLV treatment (Farag and Pare, 2002; Engelberth et al., 2004), this is the first reported genetic evidence were GLV-deficient mutants show a reduction in VOC production. This important finding demonstrates the role that within-plant GLVs play in the regulation of herbivore-induced VOCs. Whether or not GLVs have a direct impact on VOC production or if GLV induced VOC production is JA mediated remains to be determined; nevertheless, our results suggest that within-plant GLVs elicit VOC emissions during BAW feeding. Conclusive evidence demonstrating the definitive interaction between GLV, JA, and VOCs will be obtained following our creation of other key mutants and/or double mutants in the respective pathways.

As one would expect, the reduction of direct and indirect defense responses would result in increased plant susceptibility to insects. With the already well established roles of GLVs, JA, and VOCs in defense response to herbivory, we tested lox10 mutants for any alteration in their resistance to BAW. Our data suggests that deficiencies in LOX10-derived metabolites results in significantly impaired resistance (Figure 7). Our BAW preference data likewise suggests that GLVs and/or JA are significant deterrent factors in maize. These results, coupled with the additional evidence that *lox10-2* and *lox10-3* mutant plants had a significant decrease in fresh weight gain after BAW feeding, establishes the biological function of LOX10 in resistance to insects.

Based on the finding that LOX10-derived GLVs regulate JA- and VOC-mediated direct and indirect defense responses, respectively, for resistance to insect attack, a model is proposed that describes this interaction (Figure 8). It is proposed that linolenic acid is catalyzed by LOX10 to produce 13-HPOTE, which is fed to the HPL branch of the LOX pathway for GLV production. Upon wounding/herbivory, GLVs released from damaged tissue induce transcription of *ZmLOX8* and *ZmOPR7/8* for increased levels of JA production. This data coupled with the results showing reduced levels of JA in *lox8* mutants, suggests that LOX8 is the isoform responsible for wound-induced JA in maize. Collectively, the data also strengthens the role for OPR7/8 in wound-induced JA production. Because both exogenous GLVs and JA have been proposed to induce VOC emissions (Farag and Pare, 2002; other), it is further suggested that GLVs and/or JA amplify

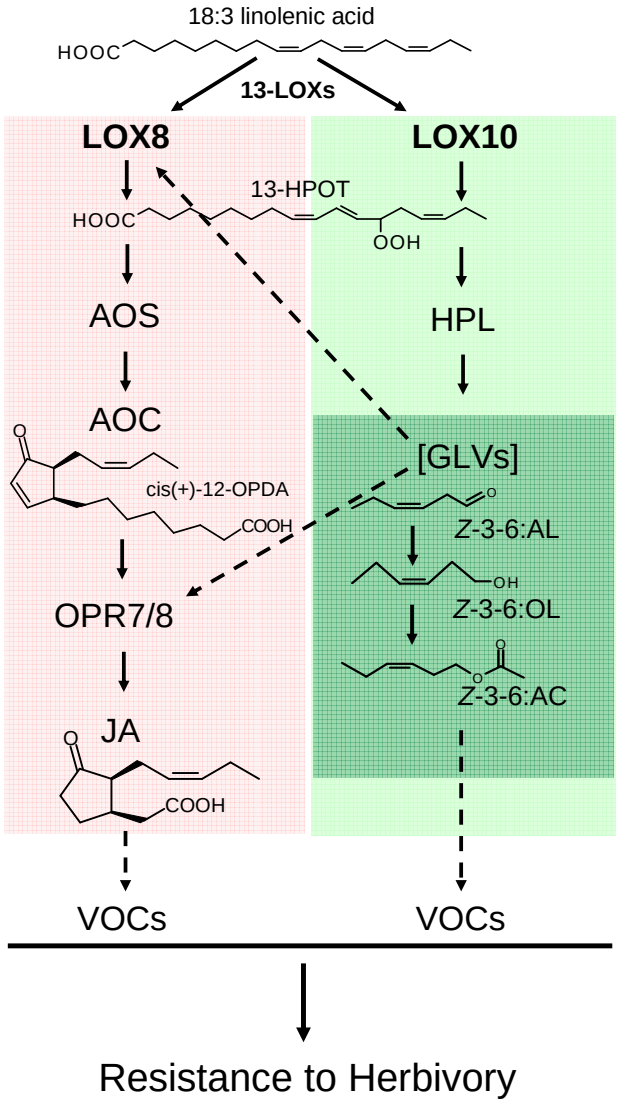


Figure 8. A working model for the role of ZmLOX10 in GLV biosynthesis and the regulation of JA and VOCs. AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, oxophytodienoic acid reductase; HPL, hydroperoxide lyase. Solid black arrows indicate known mechanisms, dashed black arrows designate the hypothetical role of GLVs in the transcriptional regulation of JA biosynthesis enzymes (ZmLOX8 and ZmOPR7/8) and VOC production.

VOC emissions for indirect defense. The collective induction of these important defensive compounds results in conferred resistance to herbivory.

In summary, we have provided strong genetic evidence that LOX10 is the maize 13-LOX required for GLV biosynthesis and have shown by our results, that LOX10-mediated GLVs regulate JA and VOC production. A direct result of LOX10s function in GLV, JA, and VOC production is evident by the susceptibility levels of the mutants to BAW feeding during the described biological assays in this study. These findings denote *ZmLOX10* as an important herbivore defense-related gene and open the way for additional elucidation of herbivore-induced defense mechanisms in maize.

**Methods**

Generation of *lox10-2* and *lox10-3* mutant alleles

The reverse genetics resource [Trait Utility System for Corn (TUSC)] at Pioneer Hi-bred Inc. was used to generate mutant alleles as described by Gao et al. (2007), using the *Mu* (*Mutator*) specific primer 9242 (5′-AGA-GAA-GCCAAC-GCC-AWC-GCC-TCY-A-3′) and the following *ZmLOX10* gene-specific primers (61560 F 5'-CGC-TAG-CTT-AGC-CAC-CAG-TAG-TCC -3'; 61561 R GCG-CTG-GAA-GTA-CAT-CTG-CCC-GA; 61562 R GGA-AGC-GCA-GAT-CCT-TCT-TGT-TGA-TGA-G; 61563 F GCC-ATC-GGG-CAG-ATG-TAC-TTC-CAG; 61564 R TAG-TCG-TAG-

ACG-CGC-TCG-AAC-ACC-TT; 61565 R AAC-TCC-TCG-TCC-TTG-AAC-CAC-GAG-AAC; 61566 F TTC-TCG-TGG-TTC-AAG-GAC-GAG-GAG-TTC; 61567 R CCT-CTG-CAT-GCA-ATA-ATA-GTA-CAC-CCC-C; 61568 F CGG-CTG-TTC-ATC-CTG-GAC-TAC-CAC; 61569 R CAG-GTT-GTT-GTT-GCA-CTC-GTC-GAT-GAA-C). Three *Mu*-insertional alleles were detected for the *ZmLOX10* locus (*lox10-1*, *lox10-2* and *lox10*-3) and the flanking regions of each insertion sites were cloned with a pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced to determine the precise location of the insertion sites. The *Mu*-insertion site for *lox10-1* positioned 342 bp inside the first intron and *lox10-2* and *lox10-3* insertion sites positioned 253 and 278 bp inside the third exon, respectively. Typical of the *Mu*-insertions, the following 9 bp duplications were created: GAGTAAATT. Original mutants were backcrossed into the B73 and W438 genetic backgrounds and genetically advanced to eliminate unwanted *Mu* insertions throughout the genome and to create near-isogenic mutant and wild-type lines ($BC_2F_4$ – $BC_5F_4$; as designated below) suitable for functional analysis.

Phylogentic analysis

A maximum likelihood tree was built by aligning protein sequence from maize 13-LOXs (ZmLOX7, ZmLOX8, ZmLOX9, ZmLOX10, ZmLOX11, ZmLOX13)and other GLV- or JA-relevant 13-LOXs in tomato (TomLOXC and TomLOXD), potato (StLOXH-1 and StLOXH-3), and tobacco (NaLOX3) using Clustal W softwawre

(Guindon and Gascuel 2003). Phyologeny was reconstructed using the LG substitution model implemented in PHYLM 3.0 software.

Quantification of GLVs

To quantify GLVs from wounded plants, methods were followed as described by Engerlberth et al., (2004). Briefly, two grams of leaf-cuttings from V2 stage WT and *lox10-2* and *lox10-3* maize plants were placed into glass cylinders connected to a Super Q filter trap (Alltech Associates). Air was removed from the system through the filter at 200ml/min, ten min post-wounding. GLVs were eluted from the filter using dichloromethane, and nonyl acetate was added as a standard before analysis by GC/MS. The identity of each volatile was determined by its retention time and mass spectra relative to the standard.

Oxylipin profiling of wounded plants

WT ($BC_5F_2$) and mutant allele *lox10-2* and *lox10-3* ($BC_4F_2$ and $BC_5F_2$, respectively) maize seedlings were grown between 25-28℃ in commercial soil (Metro-Mix 366; Scotts-Sierra Horticultural Products, Marysville, OH, U.S.A.) under a 12 hour photoperiod (120 μmol $m^{-2}$ $s^{-1}$; Quantum Meter; Apogee Instruments, Logan, UT, U.S.A.). V2 seedlings were placed in the dark for a period consisting of two consecutive nights to circumvent the circadian rhythm regulated gene

expression of *ZmLOX10* (Nemchenko et al. 2006). Under green light, seedlings were wounded seven times in the mid portion of the second leaf (three wounds sites on one side of the midvein and four on the other side), using a hemostat (wound sites approx. 1-2cm apart). The second leaf from WT and *lox10-2* and *lox10*-3 seedlings were harvested in liquid $N_2$ at the 0hr, 2hr, and 12hr time points and subsequently analyzed for free oxylipins (fatty acids, JA and OPDA) as outlined in the methods from the University of Göttingen oxylipin database website (http://www.oxylipins.uni-goettingen.de/), with minor modifications. For *ZmLOX8* wound-induced analysis, a *lox8* mutant (known as *ts1-ref* allele of *ZmLOX8*) was acquired from the Maize Genetics Cooperation Stock Center at The University of Illinois at Urbana-Champaign (Maize COOP, http://maizecoop.cropsci.uiuc.edu) as a segregating 1:1 heterozygous: mutant population. Heterozygous individuals were selfed and a subsequent 1:2:1 segregating population was grown under light shelves (120 µmol $m^{-2}$ $s^{-1}$; Quantum Meter; Apogee Instruments, Logan, UT, U.S.A.) at room temperature (22-24℃) until about V -3 stage. Leaf tissue was wounded as described above and collected 1hr post treatment. JA was measured by LC/MS in negative phase mode using methods outlined by Pan et al., (2008). In brief, ~100mg of leaf tissue was placed in a 2-ml screw-cap Fast-Prep tube (Qbiogene, Carlsbad, CA, U.S.A.) with 10-50ng of the standard dihydro JA and 0.5-1g of Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, U.S.A.). Samples were homogenized by a Precellys® 24 tissue homogenizer (Bertin Technologies, Saint-Quentin-en-Yuelines-Cedex, France)

and shaken for 30min at 4ºC. Dichloromethane was then added (1ml) and then samples were reshaken for 30min at 4ºC, followed by centrifugation for 5min at 13,000g. The bottom organic phase was then transferred into 1.8ml LOV autosampler glass vials (WVR International, West Chester, PA), evaporated by continuous air flow, and then dissolved in 300ul of methanol. JA was measured on a QTrap 2000 (Applied Biosystems, Foster, CA) LC/MS in multiple reaction mode, using a Discovery C18 HPLC column (5 cm×2.1, 5µm particle size; Supelco, Bellefonte, PA) at a 100ul/min flow rate.

Northern blot analysis

For northern blots, total RNA was extracted using the standard TRI reagent protocol (Molecular Research Center Inc., Cincinnati, OH). Following extraction, RNA (12ug) was separated by a 1.5% formaldehyde/1X MOPS gel and transferred to a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, MA) in 10XSSC (1500 mM sodium chloride, 150 mM sodium citrate) overnight. Blots were prehybridized with UltraHyb hybridization solution (Ambion, Austin, TX) and probed overnight with a $^{32}$P-labeled *ZmLOX10, ZmLOX8,* or *ZmOPR7/8* gene-specific probe as indicated below. Blots were washed 2 X 5min (or 2 X 15min for *ZmLOX8*) with a 2×SSC and 0.1% SDS (sodium dodecyl sulfate) solution (an additional 2 X 5min or 3 X 15 min wash with 0.1×SSC and 0.1% SDS was performed for the blots hybridized to *ZmLOX8* gene specific and *ZmOPR7/8*

probes, respectively) and exposed to BioMax X-ray film (Kodak, Rochester, NY) at –80 ºC for 1 to 6 days prior to developing the films. rRNA loading controls were visualized with ethidium bromide staining and UV transillumination. For gene expression of *ZmLOX8* and *ZmOPR7/8* in both WT ($BC_4F_2$) and *lox10-2* and *lox10-3* ($BC_4F_2$ and $BC_3F_7$, respectively) mutant backgrounds, seedlings were grown at 25-29℃ in commercial soil (SB300 Universa l Mix; Sungro Sunshine, Belle Vue, WA, U.S.A.) under a 12 hour photoperiod. V-2 stage plants were wounded using the treatment described above and immediately harvested in liquid $N_2$ at 0 h, 30 min, 1 h, 2 h, 4 h and 8 h for northern blot analysis.

Beet armyworm volatile collection and biological assays

Preference and performance feeding assays were carried out with WT and *lox10-2* and *lox10-3* seedlings in the B73 genetic background. Five replicates, each with seedlings planted in 5-inch pots, were randomly arranged to have treated and untreated lines consisting of individual WT, *lox10-2* or *lox10-3* seedlings (performance test) or all three genotypes together (preference test) in each replicate. When seedlings reached the V-3 stage, the three plants were loosely put together to form a trio. A cage sleeve was then carefully placed over the pot/plants and an individual $2^{nd}$ instar BAW was weighed and placed in the middle of the trio before the cage was fastened at the top. Six days post infestation, BAW were removed and weighed to determine total weight gain and seedlings were cut

at the soil level and fresh weight was taken. For VOC emission collections, BAW were reared on pinto-based artificial diet at 25˚C to the 3rd instar developmental stage. The night before volatile collection, BAW were fed in individual diet cups on corn leaf cuttings. Experimental treatments (consisting of infested and uninfested WT and *lox10* mutant plants) and sample extractions were carried out in the J. Tumlinson lab at Penn State University, as previously described by Cardoza et al., (2006).

# CHAPTER III

# LOX10-MEDIATED METABOLITES CONTRIBUTE TO MAIZE SUCCEPTIBILITY TO ANTHRACNOSE LEAF BLIGHT AND STALK ROT AND AFLATOXIN CONTAMINATION OF SEED

## Introduction

Numerous studies have attempted to characterize LOX-derived oxylipins and their roles in plant-pathogen interactions. While plant-derived oxylipins are generally thought of as defensive compounds, recent studies suggest that fungi may use endogenous plant oxylipins for their own disease development. For example, Gao et al., (2007) demonstrated that the elimination of LOX-derived host oxylipins caused drastic reductions in conidiation and mycotoxin production by the maize pathogen *Fusarium verticillioides*. Another report proposed that host-derived oxylipins may mimic the mycotoxin-inducing function of fungal psi- (precocious sex inducer) factors to regulate the expression of psi producing oxygenase (ppo) genes and thus regulate mycotoxin production (Tsitsigiannis and Keller, 2007). The general goal of this study was to broaden our knowledge of oxylipin signaling as it pertains to 13-LOX-derived oxylipins and their interactions with pathogens in maize.

In the previous chapter, the function of LOX10 in GLV biosynthesis and herbivore-related defense responses was established. In this project, I tested

whether LOX10-derived GLVs would similarly mediate plant-pathogen interactions. Existing evidence implicate GLVs in microbial defense response. For example, E-2-hexenal has been demonstrated to have strong anti-bacterial properties because of the presence of an α,β-unsaturated carbonyl group in the structure of its molecule. This carbonyl group forms adducts with nucleophiles through Michael-type addition reactions, which affect protein cross-linking and the sulfhydryl groups essential for protein function (Croft et al., 1993; Almeras et al., 2003). GLV-mediated microbial defenses are not limited to bacterial infections. Two reports provide evidence suggesting that GLVs have fungicidal effects (Prost et al., 2005; Matsui, 2006). In addition to previous pharmacological studies, recent genetic evidence demonstrates that *Arabidopsis* plants overexpressing HPL were more resistant to *Botrytis cinerea* while the antisense HPL lines were more susceptible (Shiojiri et al., 2006).

Despite the vast majority of studies that advocate GLVs as having defense related effects against microbial pathogens, one study provided evidence suggesting that they may act as susceptibility factors. Fallik et al. (1998) showed that low levels of GLVs enhanced *Botrytis cinerea* germination in vitro and on strawberries, which led to the hypothesis that pathogens may use GLVs emitted by damaged plant tissue as signals to germinate (Matsui, 2006). In order to test the role of LOX10-mediated GLVs in plant pathogen interactions, I utilized GLV-deficient *lox10* mutants (Chapter II), to test for resistance to the two economically

important fungal pathogens of maize, *Aspergillus flavus* and *Colletotrichum graminicola*.

*Aspergillus flavus* is a necrotrophic pathogen that infects oil rich seeds (i.e. peanuts, tree nuts, and corn) for their pools of lipids, mainly triglycerides, that they utilize as carbon sources for growth and development (Brodhagen and Keller, 2006). *Aspergillus flavus* accounts for devastating annual losses in corn production as a result of its ability to contaminate seed with carcinogenic mycotoxins (i.e. aflatoxins; AF) that cause liver cancer and immunosuppression in animals and humans. *Colletotrichum graminicola* is also a serious maize pathogen that causes anthracnose leaf blight and stalk rots, which account for 5-10% (1.05-2.1 billion dollars) of losses in maize production each year. To determine if LOX10-derived metabolites play a defensive role in resistance to these two important pathogens, we tested for (1) levels of vegetative growth, conidiation, and aflatoxin accumulation in response to *A. flavus* infection; and (2) severity in disease symptoms for anthracnose leaf blight (ALB) and anthracnose stalk rot (ASR) after *C. graminicola* infection.

## Results

*lox10 mutants are more resistant to contamination with aflatoxins*

To determine whether LOX10 has a function in vegetative growth, conidiation, and AF accumulation, I ran in-lab kernel assays and carried out two years of field based testing of WT and *lox10* mutants. No differences were observed for vegetative growth and conidiation between WT and *lox10* mutants in the lab based kernels assays. In the field trials, each genotype was represented by four rows (each row considered as one replicate). WT and mutants (~15 plants per row) were arranged amongst other rows of varying genotypes in a randomized complete block design. To minimize inherent variability often observed in field testing, individual samples from each replicate were harvested and pooled together (Betrán and Isakeit 2004). In the College Station 2006 trial, *lox10-3* mutant seed AF accumulation was 69% lower than WT levels, but differences were not statistically significant (Figure 9). In College Station 2007, *lox10-3* mutant levels were 94% lower than WT AF levels ($P<0.0001$). These results suggest that the LOX10-mediated lipid metabolic pathway may be responsible for greater levels of AF accumulation in seed under field conditions.

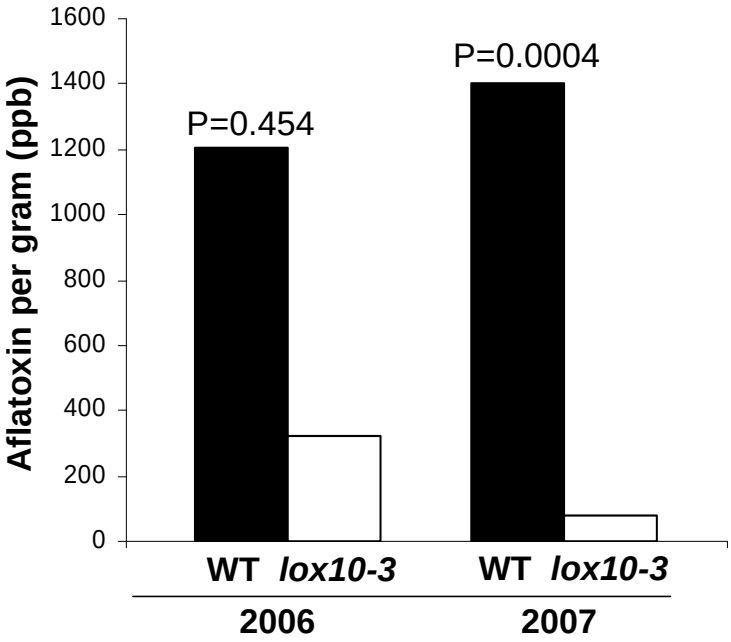


Figure 9. Aflatoxin biosynthesis in *lox10-3* mutants in College Station 2006 and 2007 fields.

Four rows of *lox10-3* mutants (~20 plants per row) were arranged in a randomized complete block design consisting of 84 total rows. Half the plants in each row were inoculated 10 days after mid-silking with 3ml of $3X10^7$ spores in liquid suspension, while the other half was left uninoculated. After ears were harvested, ground, and bulked, aflatoxin levels were quantified from 50g sub samples with a VICAM aflatest fluorometer USDA-FIGS protocol; (mean ± SD; One-way ANOVA; *** P≤ 0.001)

*lox10* mutants are more resistant to *C. graminicola*

To further examine the role of LOX10 in organs other than seed, *lox10* mutants and WT were tested for resistance to anthracnose leaf blight (ALB; a leaf disease) and anthracnose stalk rot (ASR; a stem disease) caused by *C. graminicola*. For ALB, WT and *lox10-3* mutant leaves were inoculated by the drop inoculation technique and monitored for the development of disease symptoms such as lesion area. Figure 10A shows a clear difference in the severity of ALB infection between WT and *lox10-2* mutant plants. Quantification of disease symptoms demonstrates that *lox10-2* mutant lesion areas were significantly reduced compared to WT (Figure 10B; $P \leq 0.0001$).

Similar to leaves, *lox10-3* mutants show greater resistance than their counterpart WT plants in stalk rot assays (Figure 11A). Eighty percent of the measured lesion areas in *lox10-3* stalks were < 200mm$^2$, while approximately 80% of the WT had lesion areas > 200mm$^2$, 45% of which were >400mm$^2$. A clear distinction in infection levels is depicted in Figure 11B, where the lesion areas in WT stalks are clearly larger than those of *lox10-3* mutants. These data suggest that LOX10-derived metabolites enhance susceptibility to *C. graminicola* in multiple plant organs.

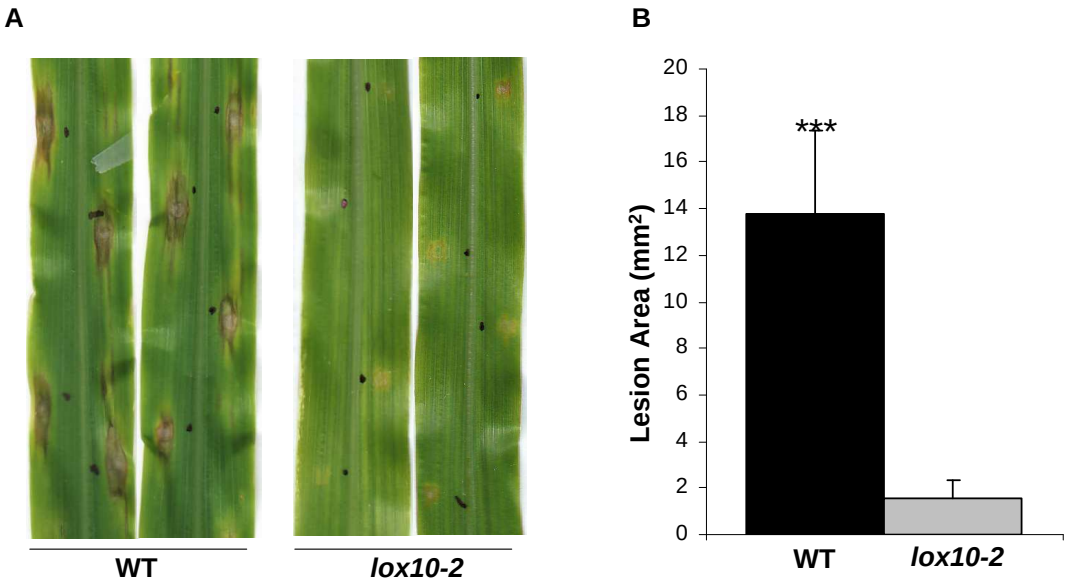


Figure 10. *lox10-2* mutants are more resistant to anthracnose leaf blight.

(**A**) Wild-type and *lox10-2* mutant plants in the W438 genetic background were drop inoculated with 10ul of *C. graminicola* (strain M1.001) spore suspension ($10^6$). Four days post inoculation, leaves were harvested and scanned to produce digital images. Lesion area was measured using the software ImageJ. *lox10-2* plant disease symptoms are less severe than wild-type.
(**B**) Lesion areas ($mm^2$) from scanned images were measured in WT and *lox10-2* mutants in the W438 genetic background. (mean ± SD; One-way ANOVA; *** P≤ 0.001)

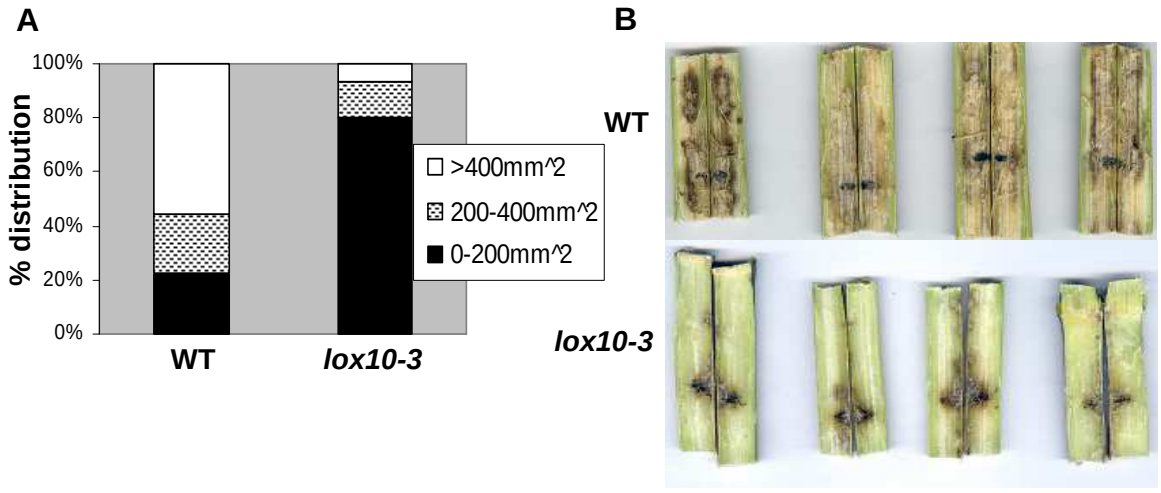


Figure 11. *lox10* mutants are more resistant to anthracnose stalk rot in the B73 background.

**(A)** Lesion areas ($mm^2$) were quantified 10 days post infection with ImageJ software. Measured lesions areas were grouped into the following three categories: 0-200 $mm^2$; 200-400 $mm^2$; >400 $mm^2$.
**(B)** Images of disease symptoms on WT and mutant stalks from infection with *C. graminicola*– Pictures were taken 10 days post-infection. Lesion areas in *lox10-3* mutants are notably smaller than WT.

Pretreatment with purified GLVs enhances disease symptoms

Because previously published data implicated GLVs in defense rather susceptibility, our surprising results showing that *lox10* mutants were more resistant to *C. graminicola* suggested that GLVs may facilitate pathogenicity in this specific host-pathogen system. To further test whether the *lox10* mutant phenotype is due to GLV deficiencies, we pretreated plants with GLVs and then infected with *C. graminicola*. GLV-pretreated plants exhibited 44% larger lesion areas than the control plants (Figure 12A and B; $P<0.001$). Collectively, the anthracnose leaf blight bioassays suggest that LOX10-mediated GLVs may have a stimulating effect in disease development for *C. graminicola*.

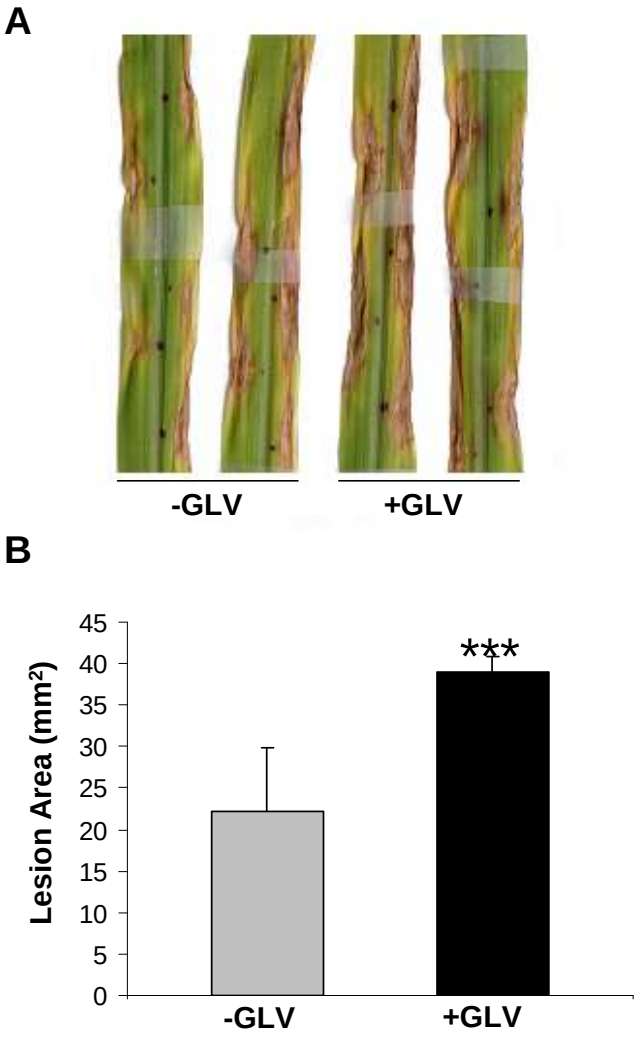


Figure 12. Pretreatment with exogenous GLVs increases susceptibility of B73 seedlings to anthracnose leaf blight.

(A) Anthracnose leaf blight disease symptoms on WT maize plants. Plants were exposed to GLVs (hexenyl acetate, 3.33ug/L) overnight and then infected with *C. graminicola* spore suspension ($10^6$). Image depicts lesions as seen five days post infection.
(B) Analysis of lesion area ($mm^2$) from leaves treated with or without GLVs. (mean ± SD; One-way ANOVA; *** P≤ 0.001)

## Discussion

To withstand diverse environmental stresses, plants evolved complex signaling networks that interact to facilitate proper responses to specific stress factors. While the detailed molecular and biochemical regulation of these responses is still obscure, it is widely known that much of the essential components involve products from the fatty acid signaling pathways including metabolites derived from LOXs. As evidenced by several recent studies, some of the LOX-derived oxylipins implicated in defense responses are GLVs. Despite the published records that advocate GLVs as having antimicrobial activities, my results suggest that LOX10-derived metabolites, most likely GLVs, may facilitate pathogenicity processes in the two fungal pathogens, *A. flavus* and *C. graminicola*. The fact that *lox10* mutants were unexpectedly more resistant to AF accumulation, ALB, and ASR may be a result of a deficiency in LOX10-derived host factors for which the pathogens have evolved a dependency. Alternatively, the increase in susceptibility for WT plants may be due to LOX10-mediated signals that mimic the function of endogenous fungal oxylipins known to regulate pathogenicity. Because *A. flavus* and *C. graminicola* infect different organs of plants, the potential mechanisms underlying increased resistance in *lox10* mutants may also be different. Therefore, we present possible explanations for each host-pathogen system based on the published literature.

One potential mechanism for the apparent inability of *A. flavus* to produce aflatoxin in *lox10* mutants is that LOX10-derived oxylipins may mimic the function of endogenous oxylipins required for normal levels of mycotoxin production. The genes known to mediate AF biosynthesis are cyclooxygenase-like psi producing oxygenases (i.e. ppoC), the deletion of which results in the elimination of sterigmatocystin (ST; a penultimate precursor of AF). It has been proposed, that host-derived oxylipins may mimic the mycotoxin-inducing function of fungal psi-factors to regulate the expression of ppo genes and thus regulate mycotoxin production (Tsitsigiannis and Keller, 2007). Recently, a study showed that ppo-derived volatiles (2-ethyl-1-hexanol) emitted from *Aspergillus nidulans* raised levels of AF in *Aspergillus parasiticus* by significant proportions (Roze et al., 2007). In another report it was proposed that ST production is initiated by an unknown oxylipin-like ligand that binds to a G-protein coupled receptor (GPCR) for ppo–mediated ST biosynthesis (Tsitsigiannis and Keller, 2007). These findings coupled with the evidence showing that GLV-deficient *lox10-3* mutants support significantly lower levels of AF, suggests the possibility that LOX10-derived GLVs may be the GPCR-binding oxylipins that initiate the signaling cascade for AF production.

The same pattern of reduced disease symptoms produced on *lox10* mutants is observed for *C. graminicola* infected maize plants. In the ALB assays, lesion areas were visibly larger on WT leaves as compared to mutants, which was consistent with the phenotype seen in stalks. Again, we hypothesize that GLVs might be responsible for greater susceptibility of WT. GLV emission upon fungal

infection has been previously reported (Cardoza et al., 2002), and while GLVs are generally perceived as defense compounds against fungal species, their role as putative stimulants for fungal growth and infection has been demonstrated through induced increases in *Botrytis cinerea* growth on strawberries (Fallik et al., 1998). The hypothesis that GLVs may contribute to susceptibility to some fungi was further strengthened by the results showing that exposure of plants to exogenous GLVs resulted in increased disease severity.

The results suggesting that GLVs may facilitate pathogenicity by the two fungal species tested in this study, raised a question about the possible mechanisms behind this previously unreported effect of GLVs. One potential metabolite known to be regulated by GLVs is JA (see Chapter II). Since GLVs are required to produce normal levels of JA in maize, and Gao et al. (2009) showed that *lox3* mutants, which are more susceptible to *A. flavus* and contamination with AF, accumulate greater levels of JA, it is feasible that GLV-regulated JA may play a role in maize susceptibility to *A. flavus*. A recent report showed that *Fusarium oxysporum* was able to hijack the JA signaling pathway through interaction with COI1 to foster disease in *Arabidopsis* (Thatcher et al., 2009). To test whether or not the JA signaling pathway or its derivatives have a function in *C. graminicola* disease progression, I infected WT and *lox10* mutant seedlings and measured JA at various time points. The results showed very low levels of JA in both WT and *lox10* mutant seedlings (data not shown), which suggests *C. graminicola* infection

may suppress JA production, but further experiments are needed to confirm this possibility.

In summary, I have demonstrated that the maize 13-LOX, *ZmLOX10*, plays an unexpected role in promoting disease development in *A. flavus*-maize and *C. graminicola*-maize interactions. These results indicate that LOX10-derived GLVs may facilitate aflatoxin accumulation in response to *A. flavus* infection. Moreover, my results demonstrate that *lox10* mutants are more resistant to ALB and ASR caused by *C. graminicola* and show that GLV-pretreated plants have more developed disease symptoms than non-treated controls. Collectively, these results suggest that LOX10-derived GLVs may be susceptibility factors to at least the two common maize pathogens tested in this study.

**Methods**

Analysis of AF levels under field and lab conditions

AF accumulation in maize kernels under field conditions was observed over two consecutive years (2006 and 2007) at the Texas Agriculture Experiment Station in College Station, TX. Four replicates per genotype were planted in a random bock design. Replicates consisted of approximately 10-20 plants per 7.3m row, with 76cm row spacing and 30cm plant spacing. Ten days post mid-silking, half the primary ears in each replicate were inoculated with 3ml of *A. flavus* NRRL 3357

suspension ($3X10^7$ conidia; Betrán and Isakeit 2004; Wicklow et al., 1998; Windham et al., 2003) by employing the non-wounded silk channel method (Zummo and Scott 1992); whereas, the other half of the plants were left nontreated as controls. Primary ears were collected at crop maturity when moisture levels were below 15% and kernels from each replicate were shelled, bulked, and ground with a Romer Mill (Romer Labs, Union, MO, U.S.A.) in preparation for AF analysis. AF levels were determined by extracting a 50g subsample with 80% methanol using monoclonal affinity columns and fluorescence determination with the Vicam Aflatest (Vicam, Watertown, MA, U.S.A.) as described in the protocol from the United States Department of Agriculture Federal Grain Inspection Service (USDA FIGS; United States Department of Agriculture 1997).

Leaf treatments with *C. graminicola* and GLVs

To perform *C. graminicola* leaf blight assays, methods were followed as described in Gao et al., (2007) with minor modifications. In brief, WT and *lox10-2* mutant maize plants were grown for 2-3 weeks in sterile Metro-Mix 366 soil (Scotts-Scierra Horticulture Products, Marysville, OH, U.S.A.) under a 16hr photoperiod (120 µmol $m^{-2}$ $s^{-1}$; Quantum Meter; Apogee Instruments, Logan, UT, U.S.A.) at 23˚ C. Plants were placed horizontally in trays lined with paper towels and inoculated with five to seven 10ul droplets of *C. graminicola* M1.001 suspension

($10^6$ conidia/ml). Following inoculation, the paper towels in each tray were wetted with 250ul of distilled $H_2O$, covered with Press-N-Seal (The Glad Products Company, Oakland, CA, U.S.A.), and incubated at room temperature. Twenty-four hours post inoculation, plants were uncovered and allowed to dry (~5-6hrs) before placing in a vertical position. Five days post inoculation, the third leaf from each plant was harvested and scanned. Lesion areas were measured using ImageJ software (ImageJ 1.36b; Wayne Rasband, NIH, Bethesda, MD, U.S.A.).

For GLV treated experiments, WT plants were grown at 22-24℃ in commercial soil (SB300 Universal Mix; Sungro Sunshine, Belle Vue, WA, U.S.A.) under a 16hr photoperiod (120 μmol $m^{-2}$ $s^{-1}$; Quantum Meter; Apogee Instruments, Logan, UT, U.S.A.). Two- to 3-week-old plants were sealed for 15hrs in 16.8L glass containers with a cotton swab containing 56ul of either hexenyl acetate diluted in EtOH (1ug/ul) or just EtOH. Following GLV treatment, plants were inoculated and disease symptoms were measured as described above.

Stalk rot assays with *C. graminicola*

For stalk rot assays, methods were again followed as described by Gao et al. (2007) with minor modifications. Stalks of 10-week-old maize plants grown in a green house (22-28˚C) were pierced with a needle (.55cm in depth) and then inoculated with *C. graminicola* 1.001 by dipping a cotton swab in suspension (around 150ul; $10^6$ conidia/ml), applying it to the wound site, and wrapping it with

Parafilm (Pechiney Plastic Packaging, Chicago, IL) to create a humid environment. Following the inoculation of three internodes on each plant, stalks were incubated for 10 days before harvesting. Harvested stalks were split longitudinally, scanned, and lesion area was measured as described above.

# CHAPTER IV

# CONCLUSION

The major objective of this study was to determine the biochemical, molecular and physiological roles of a specific maize lipoxygenase gene, *ZmLOX10*, with special emphasis on LOX10 derived oxylipins in plant-insect and plant-pathogen interactions. An important basis for this objective was the previously published data and my own preliminary results which lead me to my central hypothesis that LOX10 is responsible for producing the hydroperoxide substrate to the hydroperoxide lyase (HPL) branch of the lipoxygenase (LOX) pathway for biosynthesis of green leafy volatiles (GLV). Here I provided strong genetic evidence that LOX10 is the sole maize 13-LOX required for GLV biosynthesis in leaves. I further show that LOX10-mediated GLVs regulate the production of other important defense related compounds including jasmonic acid (JA) and volatile organic compounds (VOC). LOX10 involvement in GLV, JA, and VOC production is likely the basis for enhanced resistance to insects as evidenced by reduced resistance of *lox10* mutants to beet armyworm (*Spodoptera exigua*; BAW) feeding. These findings provide strong genetic and biochemical evidence of *ZmLOX10* as an important herbivore defense-related gene and underscore the significance of GLVs for plant defense mechanisms against pests.

Opposite to the defensive role that *ZmLOX10* plays in plant-insect interactions, and despite the existing evidence that advocate GLVs as having

antimicrobial activities, analyses with the two economically important maize pathogens, *Aspergillus flavus* and *Colletotrichum graminicola*, suggest that LOX10-mediated GLVs may enhance the ability of some fungi to successfully colonize host tissues and produce toxin secondary metabolites. Specifically, I provide evidence suggesting that LOX10-derived GLVs may be required for normal levels of aflatoxin biosynthesis in seed by *A. flavus*. Moreover, I demonstrate that *lox10* mutants are more resistant to *C. graminicola* and show that GLVs may contribute to maize susceptibility to anthracnose leaf blight and stalk rot diseases.

The dichotomous function of LOX10-mediated GLVs in plant-insect and plant-pathogen interactions may be explained by the coevolution of the interacting species involved. As my research indicates, pathogens may have evolved to take advantage of the GLV-mediated defense responses to insects. This phenomenon has been seen before in other plant species. For example, JA-associated defense responses are well-known for their important role in herbivore defense, yet *Fusarium oxysporum* has evidently evolved the means to hijack JA-mediated defenses to promote disease development in *Arabidopsis* (Thatcher et al., 2009). Overall, the results of this study suggest a more complex biochemical nature of plant-pathogen and plant-insect interactions than previously assumed. It is clear that additional studies to explain the molecular basis of the *lox10* mutant phenotypes, especially with regard to maize interactions with pathogens, will be required.

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