# DIVERSE FUNCTIONS OF THE TWO SEGMENTALLY DUPLICATED 9-LIPOXYGENASES ZMLOX4 AND ZMLOX5 OF MAIZE

A Dissertation

by

YONG SOON PARK

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

May 2011

Major Subject: Plant Pathology

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Approved by:

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# ABSTRACT

Diverse Functions of the Two Segmentally Duplicated 9-lipoxygenases ZmLOX4 and ZmLOX5 of Maize. (May 2011)

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M.S., Chungbuk National University, Korea

Chair of Advisory Committee: Dr. Michael Kolomiets

Plant lipoxygenases (LOX) are non-heme iron containing dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acids resulting in the synthesis of a large number of functionally diverse oxylipins. Although the physiological functions of jasmonate-producing 13-LOXs in dicots have been reported and highlighted in host defenses to pathogens and insects, the functions of 9-LOXs and 9-LOX derived oxylipins remain obscure for both monocots and dicots. The objective of this study was to elucidate the biochemical, molecular and physiological roles of a segmentaly duplicated pair of 9-LOXs, *ZmLOX4* and *ZmLOX5*, in host defenses to diverse stresses. Despite of their extreme similarities at the sequence levels, the *ZmLOX4* was preferentially expressed in underground organs, whereas *ZmLOX5* was stress-induced in aboveground organs. Both genes were highly induced by exogenous jasmonic acid (JA) but transcripts of *ZmLOX5* only were strongly induced in wounded leaves as well as in response to insect infestation, suggesting the role of *ZmLOX5* in plant resistance response against insect herbivory. To test potential function of *ZmLOX4* and *ZmLOX5*,

near-isogenic wild-type and mutants were generated. In this study, I provided genetic evidence that *ZmLOX5* is involved in host defense against insect herbivores via the regulation of wound-induced JA biosynthesis. Contrary to the role in insect defenses, *ZmLOX5* mediated metabolism contributes to enhanced susceptibility to a leaf fungal pathogen, *Colletotricum graminicola*. *ZmLOX4* appears to have evolved a defense function against *C. graminicola*. In addition, *lox4* and *lox5* mutants have opposite phenotypes in their ability to support production of conidia and to facilitate colonization of kernels in response to *Aspergillus flavus*. However, the two mutants were similar to each other in their enhanced susceptibility to kernel colonization and conidia production of *Fusarium verticillioides*. In conclusion, the data suggest that these two 9-LOXs, *ZmLOX4* and *ZmLOX5* and their metabolites have distinct roles in plant-insect and plant-pathogen interactions.

## ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Michael Kolomiets and my committee members, Dr. Starr, Dr. Shaw and Dr. Koiwa, for their guidance and support throughout the course of this research. Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience. Finally, thanks to my family in Korea and to my wife and my son for their patience and love.

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# CHAPTER I

## GENERAL INTRODUCTION

Maize (*Zea mays* L. ssp. *mays*) is a cereal grain that was domesticated in Mesoamerica and then spread throughout the American continents. Since in the late 15$^{th}$ century, maize has been successfully introduced to many countries of the world. Now, maize is widely cultivated throughout the world, however the United States remains the greatest producer of maize grain and accounts for almost half of the world's harvest. In addition to the US, China, Brazil, France, Indonesia, and South Africa are the major maize producing conturies (Bengoa, 2001). The tremendous economic importance of maize for the world makes it necessary to identify genes and pathways that may lead to novel strategies to increase yield and resistance to multiple environmental stresses. My research focuses on elucidation of the biological importance of the lipid metabolism genes with special emphasis on the lipoxygenase genes, the function of which remain poorly understood.

The plant lipoxygenases (LOX) catalyze oxygenation of polyunsaturated fatty acids linoleic acid (C18:2) and linolenic acid (C18:3), which are common substrates for LOXs (Andreou and Feussner, 2009). LOXs can be classified into two groups based on their ability to add molecular oxygen either at the 9 (9-LOX) or 13 (13-LOX) position of the carbon chain of these substrates.

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This dissertation follows the style of Plant Physiology.

The immediate products of their reaction are linoleic or linolenic acid 9- or 13-hydropeoxides, which serve as the substrates for at least seven biosynthetic pathway branches catalyzed by allene oxide synthase (AOS), hydroperoxide lyase (HPL), divinyl ether synthase, epoxy alcohol synthase, peroxygenase, and LOX itself. Many studies of LOXs and oxylipins have focused on elucidating of the role of metabolites in the AOS branch. Jasmonic acid (JA) is a final product of the AOS pathway and plays a major role in regulating both biotic and abiotic stress responses and development in plants (Glazebook, 2005; Wasternack, 2007; Baldi and Devoto, 2008; Howe and Jander, 2008). Class of better characterized oxylipins are green leaf volatile (GLV), including $C_6$ aldehydes, alcohols, and their esters produced via the HPL pathway. Accumulating evidence suggest that GLV may have antimicrobial activities (Nakamura and Hatanaka, 2002; Kubo et al., 2003) and anti-insect activities both directly and indirectly as signals (Vancanneyt et al., 2001; Leitner et al., 2005; Dudareva et al., 2006; Arimura et al., 2009).

The biological function of 9-LOXs in maize is still unclear. Therefore, the major goal of my Ph.D. research project was to identify the physiological functions of two 9-LOXs, *ZmLOX4* and *ZmLOX5* in defense responses to diverse stresses. Furthermore, it is my hope that better understanding of stress responses may contribute to increased yield and enhanced resistance to pathogens and insect herbivores. To identify the role of these two genes, near-isogenic wild-type and knock-out mutants of the two genes were generated. These lines were used for biochemical, molecular and functional analyses. I have studied the role of these two genes in host resistance or susceptibility to beet

armyworm and established their specific functions in the interactions with three major fungal pathogens of maize, *Colletotrichum graminicola*, *Aspegillus flavus* and *Fusarium verticillioides*.

# CHAPTER II

# COMPARATIVE MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF SEGMENTALLY DUPLICATED 9-LIPOXYGENASE GENES *ZMLOX4* AND *ZMLOX5* OF MAIZE

## INTRODUCTION

Plant intracellular composition of lipids is frequently altered during plant development and in response to environmental stress. This often results in the formation of the oxidized polyunsaturated fatty acids (PUFAs) known as oxylipins. Initial oxidation products, hydoperoxides of PUFAs, are generated by either auto-oxidation or by the enzymatic reactions of several enzymes including lipoxygenase (LOX, EC 1.13.11.12), α-dioxygenase and cytochrome P450 (Brash, 1999; Müller, 2004). LOXs are non-heme iron containing dioxygenases that can catalyze the regio- and stereo-selective oxygenation of linoleic acid (18:2(n-6)), linolenic acid (18:3(n-3)), or arachidonic acid (20:4(n-6)). In plants, LOXs are encoded by large gene families (Andreou and Feussner, 2009). It is often reported that individual LOX isoforms could be distinguished by their differential expression pattern, substrate specificity, and subcellular localization (Hildebrand et al., 1998; Schewe, 1998; Nemchenko et al., 2006), suggesting distinct roles in diverse physiological processes.

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This chapter is pubulished in Planta. All the experiments were carried out by myself with the exception of overexpression of recombination of ZmLOX5 (assisted by Susan Kunze and Ivo Feussner) and fall armyworm infestation (assisted by Xinzhi Ni) .

LOXs can be classified into two groups based on their ability to add molecular oxygen to either the 9 (9-LOX) or 13 (13-LOX) position of the substrate carbon chain leading to the synthesis of 9- or 13-hydroperoxy PUFAs. 9-LOXs comprise a subfamily of proteins that share relatively high amino acid sequence identity (>60%) among themselves (Vernooy-Gerritsen et al., 1984). This subfamily was named *type-1* LOXs. The second subfamily of LOXs represents 13-LOXs isoforms that can have a moderate sequence identity (~35%) to one another, harbor a plastidial transit peptide and have been designated *type-2* LOXs. To date, despite the widely recognized biological significance of oxylipins, precise physiological, molecular and biochemical function of diverse plant LOXs is still obscure.

The primary enzymatic products of LOX reaction, hydroperoxy fatty acids, are further converted to more stable oxylipins by several enzymes including allene oxide synthase (AOS), hydroperoxide lyase (HPL), divinyl ether synthase, epoxy alcohol synthase, peroxygenase, and LOX itself. Much of the oxylipin research has been focused on the AOS and HPL branches of the 13-LOX pathway. Jasmonates, including jasmonic acid (JA), methyl-jasmonic acid (MeJA) and isoleucine conjugate of jasmonic acid (JA-Ile) are produced from linolenic acid via the AOS pathway branch (Browse, 2009; Wasternack and Kombrink, 2009). Jasmonates regulate plant developmental plasticity and are key regulators of plant defense mechanisms against pathogens, insects and adaptation to abiotic stress (Howe and Schilmiller, 2002). These and other JA physiological functions are the result of interaction with other hormone-regulated signaling networks such as those mediated by salicylic acid (SA), abscisic acid (ABA),

and ethylene. Another better studied group of oxylipins, collectively called green leaf volatiles (GLV), are produced by the HPL pathway. These include $C_6$ aldehyde and $C_{12}$ ω–keto-fatty acid, $C_6$ alcohols, and their acetates. GLVs are involved in plant defenses against insects (Matsui, 2006; Howe and Jander, 2008), and have anti-fungal activity against diverse microorganisms (Prost et al., 2005).

In contrast to jasmonates and GLVs, the physiological functions of a multitude of 9-LOX-derived oxylipins are poorly understood. Available literature provides mostly correlative evidence for their role in defense to pathogens. Recent studies reported that bacteria-induced hypersensitive response (HR), a form of a programmed cell death executed by the host during incompatible interactions with pathogens, may be mediated by 9-LOXs in cotton and pepper plants (Cacus et al., 2009; Hwang and Hwang, 2009). 9-LOXs are implicated in a seed defense mechanism in response to infection by mycotoxin-producing *Aspergillus flavus* in almond (Mita et al., 2007) and maize (Gao et al., 2009). Accumulating evidence suggest that some 9-LOXs have a role in plant developmental processes. For example, disruption of a potato 9-LOX gene resulted in reduction of tuber yield, decrease in tuber size, and tuber morphological malformation, suggesting that 9-LOX generated metabolites are involved in the regulation of tuber enlargement (Kolomiets et al., 2001).

Several 9-LOX genes have been cloned from diverse monocot species such as maize, barley, rice, and wheat (Peng et al., 1994; van Mechelen et al., 1999; Wilson et. al., 2001; Mizuno et al., 2003; Agrawal et al., 2004). Relatively few 9-LOXs have been functionally studied by using gene suppression technologies in any monocot species.

Our long-term goal is to identify the functions of LOXs and their diverse oxylipin metabolites in maize development and stress adaptation. For this purpose, we have cloned 13 different maize LOX genes several of which clearly belonged to *type-1* 9-LOX subfamily (unpublished data). To date, the diverse functions of only one of the 9-LOX genes in maize, *ZmLOX3*, have been reported. By using *lox3* transposon-insertional mutants, *ZmLOX3* has been shown to be required for normal seed germination, growth and development, and defense against nematodes (Gao et al., 2008a) and in the interactions with several seed, leaf and root infecting fungal pathogens, (Gao et al., 2007; Isakeit et al., 2007; Gao et al., 2009). To further elucidate the physiological functions of other 9-LOXs, here we report on the isolation and comprehensive molecular and biochemical characterization of a closely-related pair of segmentally duplicated 9-LOX genes, *ZmLOX4* and *ZmLOX5*. This study revealed, that although these two genes share more than 94% amino acid sequence identity, they are differentially regulated at the transcript level in most organs tested and in response to treatments by stress hormones, wounding, insect herbivory and pathogen infection.

## RESULTS

### Isolation and sequence analysis of *ZmLOX4* and *ZmLOX5*

The nucleotide sequences for the *ZmLOX4* and *ZmLOX5* genes were first identified among approximately 800,000 expressed sequence tags (EST) available at the

DuPont/Pioneer and public EST databases. The two genes were represented by 17 individual EST clones for *ZmLOX4* and 63 EST clones encoding *ZmLOX5*. Sequencing of both strands of the longest EST clone, p0128.cpidd40r, representing *ZmLOX4*, revealed that only partial sequence containing 1,573 bp of the 3' portion of the gene was available. Therefore, the missing 5' portion of the cDNA was amplified by a 5'-RACE technique. The resulting full length cDNA sequence of 3,019 bp contained a complete open reading frame (ORF) of 2664 bp, 151 bp 5'-UTR and 204 bp of 3'-UTR, and was designated *ZmLOX4* (GenBank accession numbers: mRNA DQ335762, protein ABC59687). The longest EST clone representing the *ZmLOX5* cDNA, p0118.chsbc64r, was a full-length clone containing 3,085 bp with 2,664 ORF, 223 bp 5'-UTR and 198 bp 3'-UTR (GenBank accession numbers: mRNA DQ335763, protein ABC59688). The two genes encode a predicted 887 amino acids with the estimated molecular weight of 100.4 kDa (ZmLOX4) and 99.1 kDa (ZmLOX5) and an estimated pI of 6.63 (ZmLOX4) and 6.66 (ZmLOX5). The deduced amino acid sequence of ZmLOX4 and ZmLOX5 share 94% identity and ~95% homology (Fig. 1), but only ~40% to ~67% identity to other maize LOX predicted proteins. Despite their high sequence homology, *ZmLOX4* and *ZmLOX5* are discrete genes in the maize genome that are located on chromosomes 1 and 5, respectively (data not shown). These data indicate that the two genes are extremely closely related to each other and that they have likely evolved as a result of recent segmental gene duplication event.

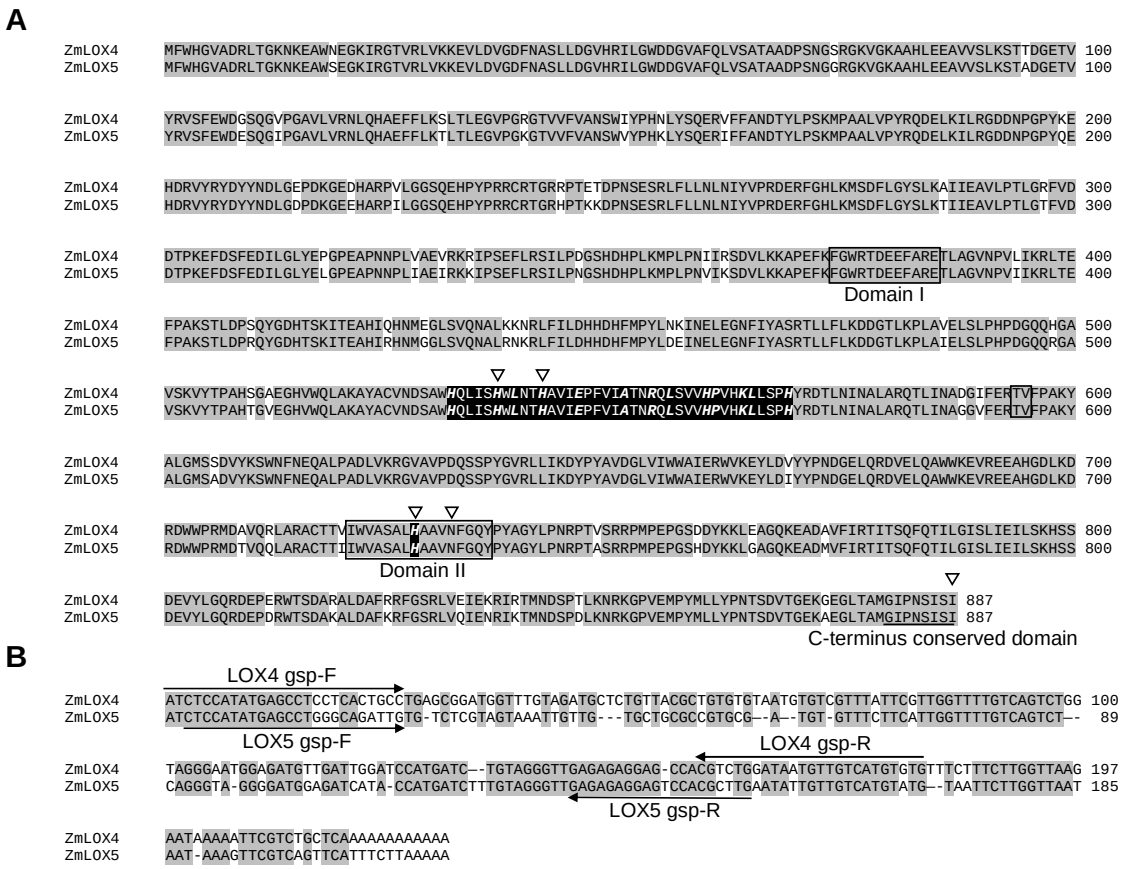


**Figure 1.** Alignment of the amino acid and nucleotide sequences of ZmLOX4 and ZmLOX5.

A, Amino acid sequence of ZmLOX4 is aligned with that of ZmLOX5 by using ClustalW. Identical residues are indicated by gray background. The conserved 38-amino acid motif required for enzyme activity is shown on black background. The 11 amino acids that accommodate the fatty acid substrates are indicated by italic font. Reverted triangles indicate the conserved amino acid residues required for iron binding and catalytic activity. Domain I, Domain II, and C-terminus conserved domain are represented as described in Gao et al., (2008b). B, Comparison of the 3'UTR nucleotide sequences used for generating gene-specific probes for the *ZmLOX4* and *ZmLOX5* genes. Primer sequences and their location are shown by arrows.

Sequence alignment of the deduced amino acid of ZmLOX4 and ZmLOX5 with other plant LOX showed that the deduced amino acid sequence of both ZmLOX4 and ZmLOX5 harbored all three conserved His residues (537, 542, and 728) and Asn732 and Ile887 (Fig. 1, reverted triangles) as well as the conserved 38-amino acid motif (Fig 1, black background) that are required for iron binding and enzyme catalytic activity in other reported plant and animal LOXs (Steczko et al., 1992; Prigge et al., 1996). Both isozymes also contained the 11 highly conserved amino acids (Fig. 1, bolded italic) that were suggested to accommodate the fatty acid substrates (Steczhko et al., 1992; Boyington et al., 1993). Previous studies identified a conserved Thr/Val motif that may determine 9-LOX regio-specific activity whereas a Ser/Phe conventional motif is believed to be an indicator of 13-LOX regio-specificity (Hornung et al., 1999). The amino acid sequences of ZmLOX4 and ZmLOX5 contained the Thr/Val motif (Fig. 1, black line), suggesting that these two LOXs belong to canonical 9-LOX subfamily. Analysis of conserved domains in the deduced amino acid of ZmLOX4 and ZmLOX5 was performed by the NCBI Conserved Domain Search program (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and Pfam software (http://www.sanger.ac.uk/Software/Pfam/search.shtml), which showed that both proteins were composed of two major domains, a N-terminal β-barrel or PLAT domain and a C-terminal catalytical LOX domain. Four different publicly available programs, including ProtComp, TargetP, ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) and WolfPsort (http://wolfpsort.org) were utilized to predict the subcellular localization of ZmLOX4

and ZmLOX5. These four programs collectively predicted that both LOXs are most likely localized in cytoplasm.

Using maize GSS and MAGI genome databases, genomic and full-length cDNA sequences of *ZmLOX4* and *ZmLOX*5 were analyzed to identify the genomic structure of these two genes. The sequence comparison showed that two genes were highly similar in the position, length, and composition of exons, and contained nine exons and eight introns.

When determining the number of closely related genes, it is important to take into account the recent discovery that maize genome of B73 inbred contains a substantial number of so called nearly identical paralogs (NIPs) (Emrich et al., 2007). NIPs are defined as paralogous genes that share ≥98% identity. Therefore, to determine whether there are any NIPs that are highly identical to the *ZmLOX4* and *ZmLOX5* genes, we performed Southern blot analysis of genomic DNA extracted from three different inbred lines using five restriction enzymes. Importantly, for this analysis the non-gene-specific probe was used that comprises 420 bp of the 3' portion of the *ZmLOX4* gene. This probe was designed specifically to hybridize to both genes as well as to other closely related genes or NIPs, because it includes a portion of the coding sequence that is highly conserved between the two genes. As Figure 2 shows, two to three bands of similar intensity were observed in the genomes of all three inbred lines tested, B73, Mo17 and A632. The lengths of the hybridized fragments corresponded closely to the restriction fragments predicted based on the positions of known restriction sites within the available

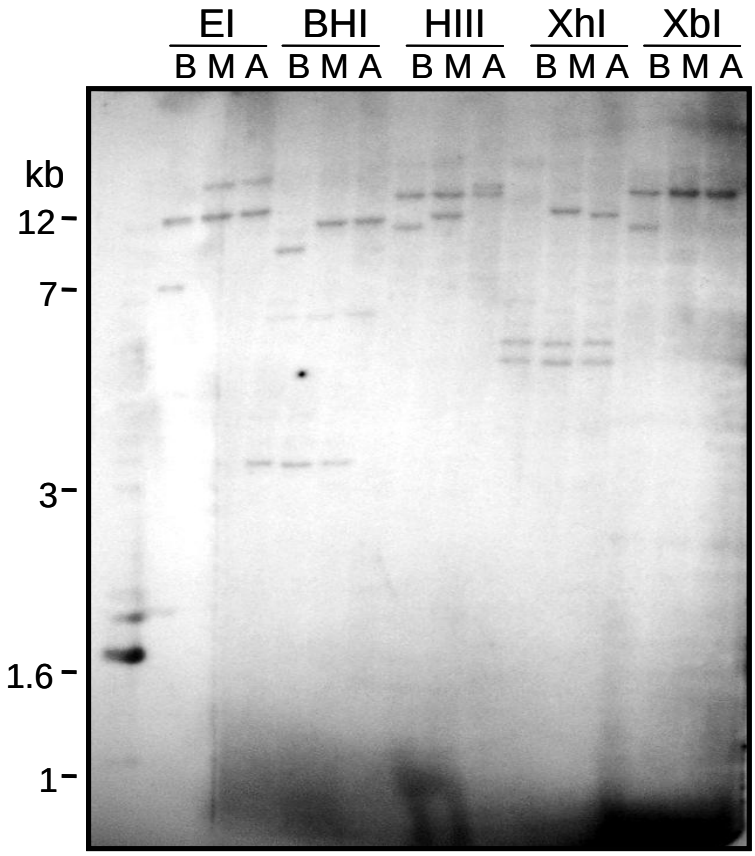


**Figure 2.** Southern blot analysis of the *ZmLOX4* and *ZmLOX5* genes in selected maize inbred lines. Maize genomic DNA (10µg a lane) of B73 (B), Mo17 (M), and A632 (A) inbred lines was digested with *Eco*RI (EI), *Bam*HI (BHI), *Hind*III (HIII), *Xho*I (XhI), and *Xba*I (XbI). DNA was separated by gel electrophoresis, blotted onto nylon membranes, hybridized with a $^{32}$P-labeled cpidd40 probe that cross-hybridizes with both genes. DNA size markers in kilobases (kb) are indicated on the left. The results show that maize genome does not contain nearly identical paralogs similar to the *ZmLOX4* and *ZmLOX5* genes.

gene sequences. These results clearly indicated that no NIP exists in the maize genome that is closely related to *ZmLOX4* and *ZmLOX5*.

The phylogenetic relationship of ZmLOX4 and ZmLOX5 to other maize and other plant 9-LOXs was investigated. This analysis revealed that monocot LOXs and dicot LOXs group together in separate clades and showed that ZmLOX4 and 5 were closely related to a single sorghum LOX gene designated SbLOXE (Fig. 3). Also, ZmLOX4 and ZmLOX5 were more closely related to rice LOX1 (OsLOX1) than to other maize 9-LOXs (Fig. 3).

**Biochemical properties of recombinant of ZmLOX5**

Since sequence similarity to other 9-LOXs is not sufficient to predict correctly whether these genes are 9- or 13-LOXs, we overexpressed ZmLOX5 recombinant protein in *E. coli* to characterize its enzymatic properties. The recombinant of ZmLOX5 protein exhibited LOX activity at two different pH conditions (pH 6.0 and pH 8.0) with linoleic acid as a substrate. HPLC analysis of the products of this reaction revealed that recombinant ZmLOX5 is a predominantly a 9-LOX because 9-HOD, 9-HPOD, and other 9-oxylipins comprised more than 92% of total oxylipins measured (Table 1). The recombinant protein is active at both neutral and slightly alkaline pH conditions. Because ZmLOX4 is 94% identical to ZmLOX5 having the same determinants for regio-specificity, we predict that *ZmLOX4* is also a 9-LOX.

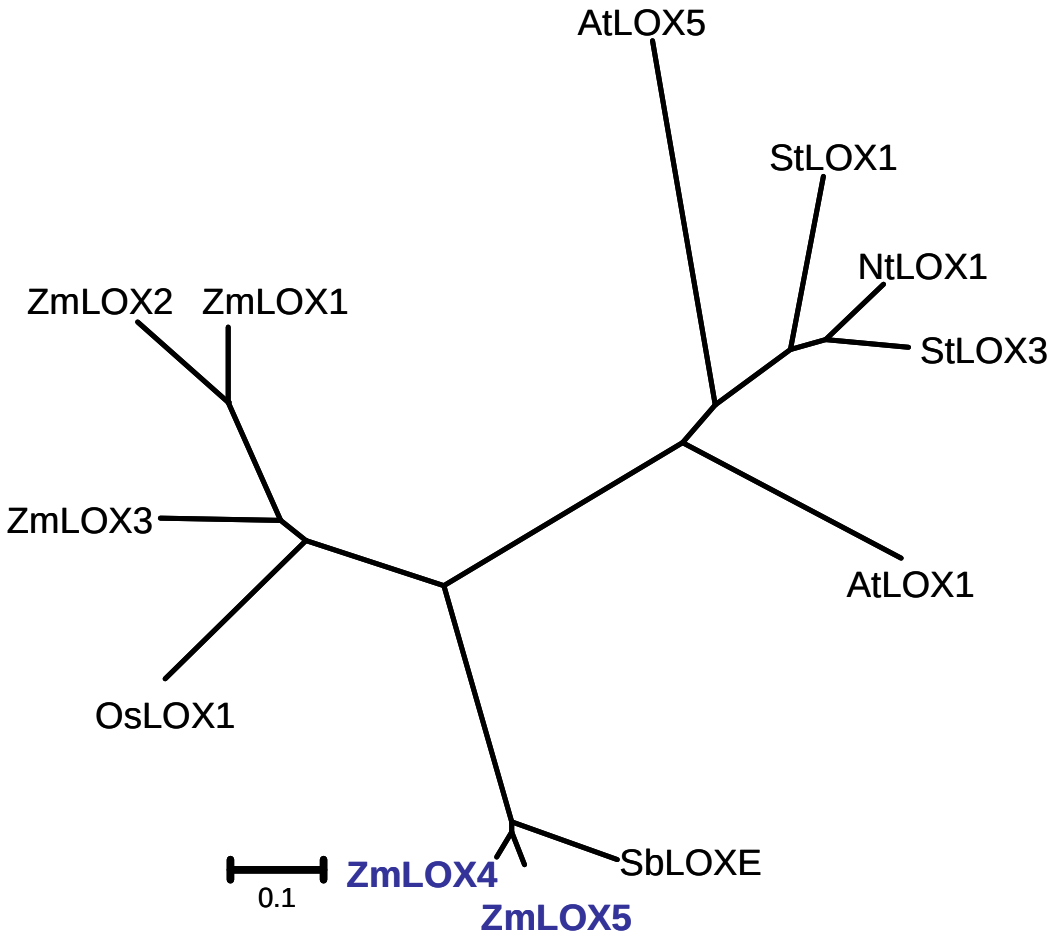


**Figure 3.** Phylogenetic analysis of ZmLOX4 and ZmLOX5 and selected monocot and dicot plant 9-LOXs. The amino acid sequence aliment of ZmLOX4 and ZmLOX5 and other 9-LOXs are carried out using Clustal W and phylogenic tree was constructed using PHYLM (version 3.0) software. *Zea mays*, ZmLOX1 (DQ335760); ZmLOX2 (DQ335761); ZmLOX3 (AF329371); ZmLOX4 (DQ335762); ZmLOX5 (DQ335763); *Nicotiana tabacum*, NtLOX1 (AAF76207); *Solanum tuberosum*, StLOX1 (AAB67858); StLOX3 (AAB67865); *Arabidosis thaliana*, AtLOX1 (AAA32827); AtLOX5 (CAC19365), *Sorgum bicolor*, SbLOXE (Sbi_0.30770:peptide); *Oryza sativa*, OsLOX1 (ABF98382).

**Table I.** Regio-specificity analysis of recombinant ZmLOX5 protein

| | Oxylipins [a] (total absorption units [b]) | | | | | |
|---|---|---|---|---|---|---|
| pH | 9-HOD | 13-HOD | 9-HPOD | 13-HPOD | other 9-oxylipins | other 13-oxylipins |
| pH 6.0 | 35166 | 1421 | 28526 | 2114 | 2668 | 2456 |
| pH 8.0 | 84413 | 2363 | 46044 | 1806 | 5388 | 6100 |

| | Oxylipins (% of total) | | | | | |
|---|---|---|---|---|---|---|
| pH | 9-HOD | 13-HOD | 9-HPOD | 13-HPOD | other 9-oxylipins | other 13-oxylipins |
| pH 6.0 | 48.6 | 2 | 39.4 | 2.9 | 3.7 | 3.4 |
| pH 8.0 | 57.8 | 1.6 | 31.5 | 1.2 | 3.7 | 4.2 |

[a] Linoleic acid was used for the assay as a substrate to measure LOX activity. Analysis of LOX activity demonstrated that linoleic acid was converted by ZmLOX5 into 9-HOD, (9S, 10E, 12Z)-9-hydroxy-10, 12-octadecadienoic acid; 13-HOD, (9Z,11E,13S)-13-hydroxy-octadeca-9,11-dienoic acid; 9-HPOD, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid, and 13-HPOD, (9Z,11E,13S)-13-hydroperoxy-9,11-octadecadienoic acid.

[b] 3736 absorption units correspond to 1 nmol of HODE.

**Organ-specific and hormone-regulated expression of *ZmLOX4* and *ZmLOX5***

Considering that the two genes are highly similar to each other throughout the entire coding sequence (Fig. 1A), for further expression analysis, gene-specific probes were generated by PCR based on a portion of 3'-UTR of these genes, which shared only 77% identity between the two genes (Fig. 1B). To test whether the probes are gene-specific, we performed a dot-blot assay. The data showed that *ZmLOX4* probe did not cross-hybridize with plasmid DNA containing cDNA of *ZmLOX5* and vice verse (Fig. 4A), suggesting that these two probes were indeed gene-specific. Therefore, these probes were used to analyze the expression patterns of *ZmLOX4* and *ZmLOX5* in all the northern blotting analyses presented in this study. Expression analyses of various maize organs showed that transcripts of *ZmLOX4* accumulated in root and shoot apical meristem but were barely detectable in crown, coleoptile, and ear (Fig. 4B). In contrast to *ZmLOX4*, *ZmLOX5* was expressed to notably greater levels in the above-ground organs including shoot apical meristem, stem, tassel, silk, and ear. Relatively low levels of the *ZmLOX5* transcripts were also detectable in the under-ground organs, crown and coleoptile (Fig. 4B). Of importance for the rest of this study is the fact that these two genes were not expressed in untreated, non-stressed leaves (Fig. 4B). Overall, expression of Z*mLOX5* was relatively higher than that of *ZmLOX4* in most tested organs and tissues except in root. This fact is further supported by three times greater number of ESTs that represent *ZmLOX5* compared to those that encode *ZmLOX4*. In summary, the data indicate that these two paralogs are differentially regulated in unchallenged maize tissues.

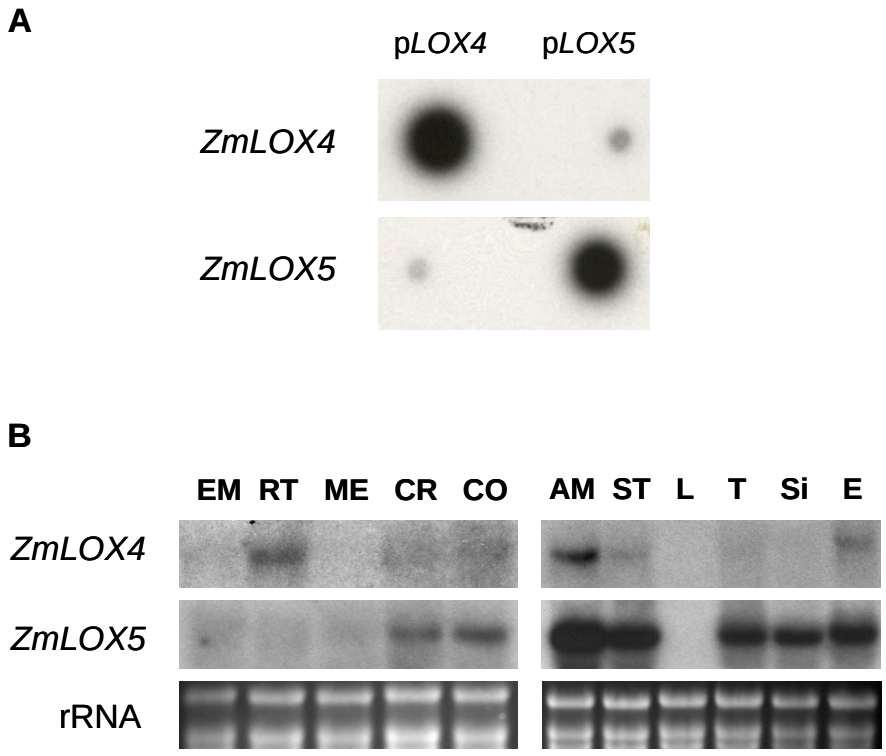


**Figure 4.** Organ-specific expression of *ZmLOX4* and *ZmLOX5*.

A, Dot blot assay showing that probes used for expression analyses are gene-specific. Gene-specific probes (gsp) were generated from the 3'UTR portions of each gene (shown in Fig. 1a) and were hybridized to 100 ng of plasmid DNA representing cDNA sequences of *ZmLOX4* or *ZmLOX5* that was denatured and dotted to nylon membranes. Results illustrate that both probes hybridized only to the cDNA fragments of the corresponding genes. B, RNA gel blot analysis of organ-specific expression of *ZmLOX4* and *ZmLOX5*. Embryos (EM), root (RT), mesocotyl (ME), crown (CR), colleoptile (CO), shoot apical meristem (AM), stalk (ST), leaf (L), tassel (T), non-pollinated silks (Si), and ear (E) were collected from B73 inbred line plants for this experiment. Stalk, leaf, tassel, silks and ear were collected from R1 developmental stage plants. Fifteen μg of total RNA was loaded into each lane of 1.5% formaldehyde RNA gel and separated by electrophoresis, transferred onto nylon membranes, and hybridized with $P^{32}$-labeled gene-specific probes. Blots were exposed to X-ray film for three days. Ethidium bromide staining of gels confirmed equal loading of RNA samples.

The hormones JA, SA, ABA, and ethylene, are implicated as signaling molecules in abiotic stress- or defense-related responses in plants (Anderson et al., 2004; Glazebrook et al., 2005; Lorenzo and Solano, 2005). To obtain clues about the potential functions of *ZmLOX4* and *ZmLOX5* in stress-induced responses mediated by these diverse signals, induction of gene expression was examined in young leaves treated with these defense-related hormones. As shown in Figure 5A, *ZmLOX4* transcripts were transiently induced by JA between 1 and 6 h after treatment. No detectable induction was observed in response to SA, ABA or ethylene. *ZmLOX5* was strongly induced by JA and SA and only slightly by ABA (Fig. 5A and 5C). Although both genes were JA-inducible, quantitative differences in expression patterns were apparent. Specifically, *ZmLOX5* transcripts accumulated to the greatest levels at 3 h, which were maintained at very high levels until 12 h after treatment with JA, whereas the levels of *ZmLOX4* remained relatively low at most time points (Fig. 5A). Ethylene appears to be the only hormone that does not alter expression of either of these two genes (Fig. 5B). Taken together, we conclude that *ZmLOX4* and *ZmLOX5* are differentially expressed in leaves in response to stress-associated hormones. These findings indicate that *ZmLOX4* and *ZmLOX5* may potentially have distinct roles in unchallenged organs as well as in stress- or defense-related responses of maize to biotic- and abiotic stresses. Intriguingly, as is notable in every control panel in Figure 5, there is a clear induction of *ZmLOX5* transcripts in mock treated control leaves.

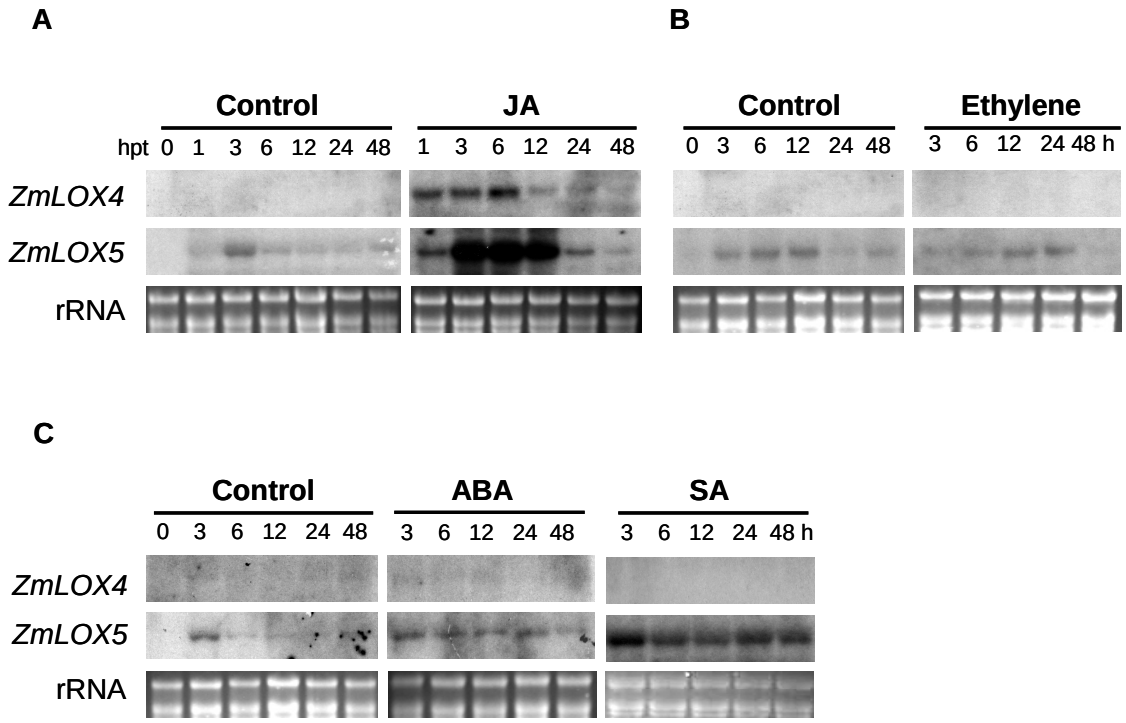


**Figure 5.** RNA gel blot analysis of *ZmLOX4* and *ZmLOX5* expression in response to defense-related phytohormones. Maize B73 seedlings (V2 developmental stage) were treated with (A) jasmonic acid (200 μM), (B) ethylene (10 μl $l^{-1}$), and (C) abscisic acid (100 μM) and salicylic acid (2.5 mM). Total RNA (15 μg $lane^{-1}$) was used for all experiments. The membranes were exposed to the X-ray film for 2 days (JA) or for 4 days (SA, ethylene, and ABA). Representative films from at least two independent tests are shown in this figure. Ethidium bromide staining of gels confirmed equal loading of RNA samples.

This interesting expression pattern implies that *ZmLOX5* may be activated, perhaps, by associated or touch stimulation similarly to that observed for a mechanosenstive LOX gene of wheat (Mauch et al., 1997).

**Local and systemic induction of *ZmLOX4* and *ZmLOX5* in response to wounding**

Wound-induced transcriptional regulation of the genes was tested by mechanical wounding of leaves and roots, and excision of stems (Fig. 6). *ZmLOX5* was transiently and strongly induced at 2 to 8 h post-treatment in wounded leaves (local response). Increased, albeit relatively low, levels of *ZmLOX5* transcripts were also detected at 3 to 24 h in systemic non-wounded leaves. In contrast, transcripts of *ZmLOX4* were barely increased in both local and systemic leaves (Fig. 6A). These results suggest that *ZmLOX5* is strongly inducible by wounding both locally and to a lesser extent in systemic, unwounded leaves, while *ZmLOX4* appears to respond to mechanical wounding of leaves only weakly.

Oxylipin analysis of the wounded leaves at 0 h, 4 h and 8 h after wounding showed that the increased expression of the 9-LOX genes in response to wounding was accompanied by elevated levels of several 9-LOX products (Table. 2).

**Table II.** Accumulation of 9-LOX-derived oxylipins in leaves in response to wounding

| | Concentration of 9-oxylipins (ng per g of FW) | | |
|---|---|---|---|
| Type of oxylipin | 0 h | 4 h | 8 h |
| 9-HODE * | 721.88 ± 260.696 | 1183.20 ± 316.332 | 1116.04 ± 304.789 |
| 9-HOTE * | 668.39 ± 224.506 | 1626.69 ± 264.204 [a] | 1303.82 ± 314.919 [b] |
| 9-HPODE * | 923.32 ± 131.536 | 1306.07 ± 330.720 | 1234.55 ± 368.643 |
| 9-HPOTE * | 900.14 ± 124.604 | 1746.99 ± 355.901 [b] | 1174.66 ± 376.403 |

* 9-HOD, 9-hydroxide of linoleic acid; 9-HPOD, 9-hydroperoxide of linoleic acid; 9-HOT, hydroxide of linolenic acid; 9-HPOT, 9-hydroperoxide of linolenic acid

Values shown are the means of five replicates ± one standard deviation at each time point.

[a, b] Differences between means were considered to be significantly different at $P < 0.05$, Different letters indicate significant differences between oxylipin contents at time 0 h and 4 h or 8 h after wounding ([a] denotes $P < 0.001$; [b] denotes $P < 0.01$ ANOVA).

Compared to the non-wounded controls (0 h), the levels of 9-hydroperoxide and 9-hydroxide derivatives of linoleic acid (9-HPODE and 9-HODE) and linolenic acid (9-HPOTE and 9-HOTE).were increased. However, the greatest and statistically significant induction was observed only for 9-HPOTE at 4 h and 9-HOTE at both 4 h and 8 h after wounding

Systemic expression of *ZmLOX4* and *ZmLOX5* was further tested in stems and leaves in response to stem excision at the soil level 3 h after the treatment, the time point at which both genes were moderately induced in systemic leaves (Fig. 6A). Consistent with organ-specific expression studies (Fig. 4B), *ZmLOX5* was expressed in stems but not in leaves of control seedlings (Fig. 6B). Interestingly, the transcript of *ZmLOX5* was strongly upregulated in stems and induced in leaves by excision of the stem, whereas *ZmLOX4* was unresponsive to the signal generated at the excision site (Fig. 6B).

Next, local and systemic expression of the two genes was elucidated in both roots and leaves in response to mechanical damage of roots. Figure 6C shows that *ZmLOX4* was expressed constitutively in undamaged roots and was further upregulated by mechanical wounding but it was not induced systemically in leaves. In contrast, *ZmLOX5* was downregulated in roots by wounding compared to non-wounded control roots at 3 h time point, but was upregulated systemically in untreated leaves of the seedlings with wounded roots (Fig. 6C). Curiously, similar to other mock-treatment controls, *ZmLOX5* was slightly induced in non-wounded roots and leaves at most time points except at 0 h, again pointing to an extreme mechanosensitive nature of the *ZmLOX5* gene expression.

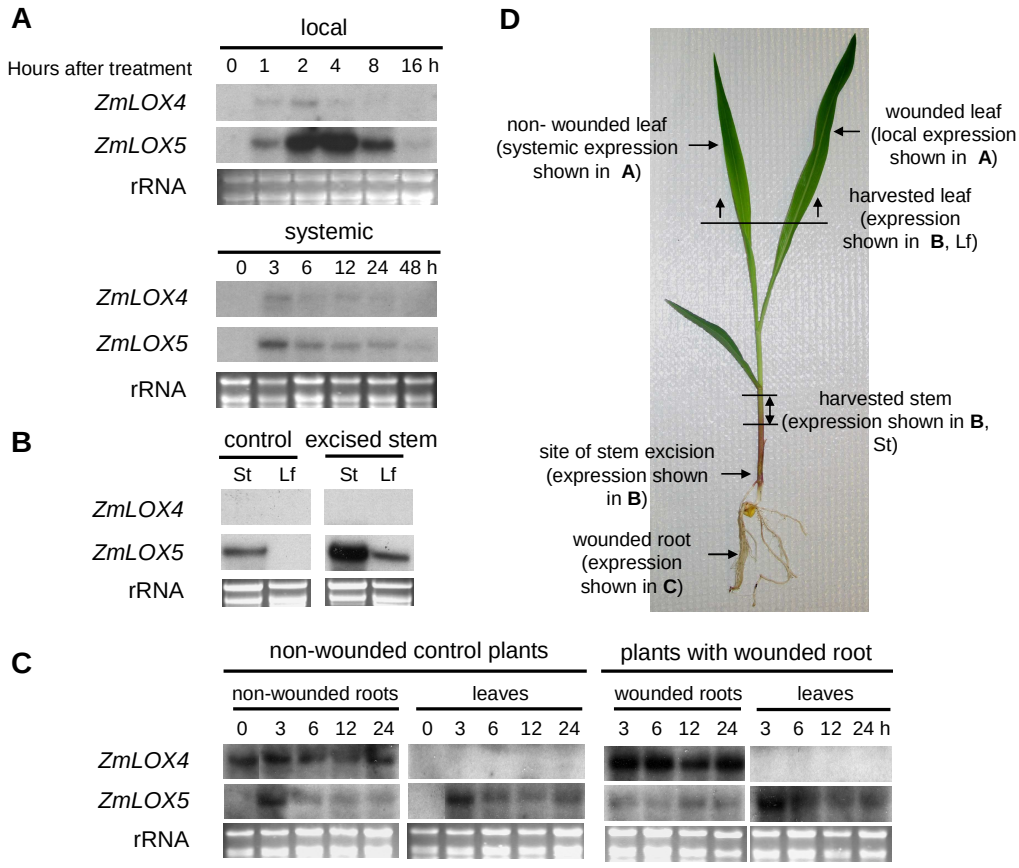


**Figure 6.** RNA blot analysis of *ZmLOX4* and *ZmLOX5* expression in response to mechanical wounding.

A, Second leaves of B73 seedlings were wounded by hemostat and RNA was extracted from both local leaves (at the wound site) and systemic (non-wounded) third leaves. Total RNA (15 μg lane$^{-1}$) was loaded into each lane. The blots showing local gene expression (upper panel) were exposed to X-ray films for 20 hours. The blots showing systemic induction were exposed to X-ray films for 7 days (bottom panel). B, Stems of V2-stage seedlings were cut at the soil level by a razor blade, placed with their cut end in water for 3 h, and gene expression was measured in the stem (St) above the cut site and in systemic leaves (Lf) as shown in the scheme D. Control plants were not excised. The blots were exposed to X-ray films for 20 hours. C, Roots of ten days-old-seedlings grown in germinating paper were wounded by hemostat and RNA was extracted from the wounded and systemic non-wounded leaves collected at the indicated time points (in hours) after wounding or from non-wounded controls. Representative blots were exposed to X-ray films for 13 days. D, Schematic representation of the experiments described in A, B and C. Ethidium bromide staining of gels confirmed equal loading of RNA samples in A, B and C.

In summary, our data suggest that *ZmLOX5* is strongly inducible in leaves and stems by wounding but not in roots, whereas *ZmLOX4* is locally induced by wounding in roots but barely responsive to wounding in leaves.

**Expression of *ZmLOX4* and *ZmLOX5* in response to insect herbivory and pathogen infection**

To elucidate the potential function of *ZmLOX4* and *ZmLOX5* in defenses against insects, the expression of these two genes was analyzed in response to infestation of leaves with fall armyworms (*Spodoptera frugiperda*). Strong induction of *ZmLOX5* was detected as early as 1 d and was maintained up to 8 d after infestation, suggesting that *ZmLOX5* may have a role in plant defense to insect herbivory (Fig. 7A). *ZmLOX4* transcripts were not induced by fall armyworm feeding, once again supporting our hypothesis that this gene has a distinct function from *ZmLOX5*.

We further tested whether *ZmLOX4* and *ZmLOX5* have a role in the plant-pathogen interactions by studying expression of these two genes in response to infection of maize leaves with *Cochliobolus carbonum*, the fungal agent causing Northern corn leaf spot. Seedlings of Pr inbred line were infected with two different strains of *C. carbonum* race 1.

*C. carbonum* tox2+ strain is the wild type strain that produces HC toxin, a pathogenicity factor for this fungus, whereas the tox2- is a mutant strain that can not produce the toxin and therefore, is not capable of causing disease. Pr inbred line is resistant to the Tox2- strain but are susceptible to the toxin producing, virulent strain Tox2+ (Multani et al., 1998). As shown in Figure 7B, *ZmLOX4* and *ZmLOX5* were only moderately induced by both fungal strains, however, transcript levels were somewhat lower during incompatible interaction with the HC-toxin deficient strain. These data indicate that expression of these 9-LOX genes may contribute to disease development during leaf colonization by *C. carbonum* in a manner similar to that reported for another maize 9-LOX, *ZmLOX3* (Gao et al., 2007). Providing evidence that the pathogen infection was successful and confirming previously published results (Nemchenko et al., 2006), a defense-related marker 13-LOX gene, *ZmLOX10,* was expressed to greater levels during the incompatible interactions (Fig. 7B).

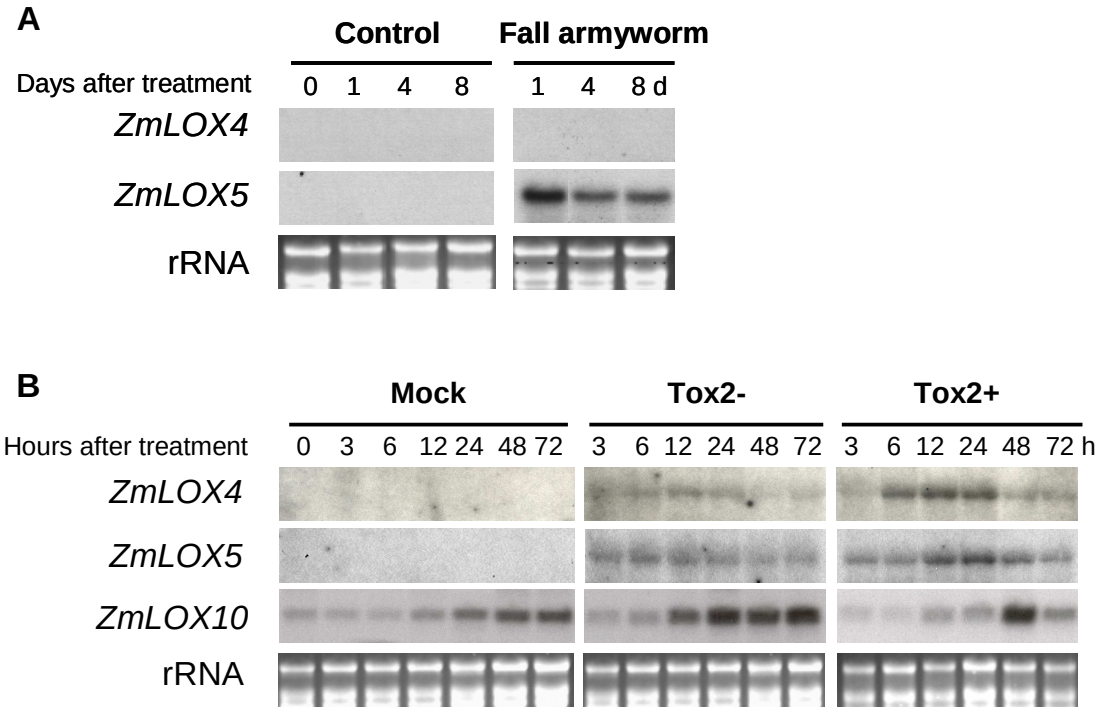


**Figure 7.** RNA blot analysis of *ZmLOX4* and *ZmLOX5* expression in maize leaves subjected to herbivory by fall armyworm or infected with *Cochliobolus carbonum.*

A, The maize plants at six leaf (V6) stage were infested with fall armyworm (*Spodoptera frugiperda*) or left uninfested for the duration of the experiment (control). Total RNA was extracted from leaves harvested at the indicated time points (days) after the beginning of infestation. Fifteen μg of total RNA was loaded into each lane, and blots were exposed for three days. B, Conidial suspension of *C. carbonum* race 1 either producing HC-toxin strain (Tox2+) or deficient in toxin production (Tox2-) was spayed on leaves of Pr inbred line at the V3 developmental stage. For the control treatment, water was sprayed onto similar age seedlings. RNA was extracted from all fully expanded leaves. The blots were exposed to X-ray films for 13 days. Ethidium bromide staining of gels confirmed equal loading of RNA samples.

## DISCUSSION

To date, our understanding of the function of a variety of 9-LOX derived products is still fragmented. Several recent studies suggested that 9-oxylipins have potent signaling activities based on their ability to induce gene expression and mediate diverse developmental and defense-related physiological processes (Kolomiets et al., 2001; Vellosillo et al., 2007; Gao et al., 2008a). Here, we report on the biochemical and molecular analysis of two maize 9-LOX paralogs, *ZmLOX4* and *ZmLOX5*. An understanding of the physiological functions of these genes requires comprehensive analysis of biochemical properties and the regulation of their expression in diverse organs and in response to diverse environmental stresses.

Since *ZmLOX4* and *ZmLOX5* are located on different chromosomes yet share high sequence similarity, we conclude that the two genes have evolved from a relatively recent segmental duplication event. Interestingly, sorghum, one of the maize closest monocot relatives, contains only one *LOX4/5*-like gene (GenBank accession Sbi_0.30770:peptide). This suggests that either maize has acquired additional *LOX4/5*-like gene or sorghum has lost one of them after their divergence from a common ancestor.

Biochemical characterization of the recombinant ZmLOX5 expressed in *E. coli* clearly defines this isoform as a predominantly 9-LOX. Since ZmLOX5 shares ~94% identity at the amino acid level with ZmLOX4 and because the amino acids that do differ are not conserved in other LOXs, we predict that ZmLOX4 is also a 9-LOX isoform.

Despite extreme sequence similarity, the two genes displayed a remarkably dissimilar expression pattern in diverse tissues and in response to stress hormones, wounding and insect herbivory. This may be explained by substantial difference in the promoter sequences of the two genes that share only 47% nucleotide sequence identity in of the region of 2 kb upstream of the transcription start site. The root- and shoot apical meristem-specific expression of *ZmLOX4*, suggests a function of this gene in the physiological processes in these organs. In fact, *ZmLOX4* expression is very similar to the expression pattern of another root-specific maize 9-LOX gene, *ZmLOX3* (Wilson et al., 2001; Gao et al., 2007). The *lox3* knockout mutants displayed reduced growth of roots and increased susceptibility to root-knot nematodes (Gao et al., 2008a) but increased resistance to a root infecting fungus (Isakeit et al., 2007). Therefore, we hypothesize that *ZmLOX4* has a role in maize interactions with various pathogens of roots. Unlike *ZmLOX4*, the *ZmLOX5* gene appears to be expressed predominantly in the aerial organs of the plant including reproductive organs with the exception of leaves. The difference in the organ-specific expression suggests that the two genes may have dissimilar roles in diverse tissues.

*ZmLOX4* and *ZmLOX5* genes are also differentially regulated by major stress hormones in leaves. While *ZmLOX4* was induced only by JA treatment, *ZmLOX5* was induced by both JA and SA. JA serves as a signal in the regulation of defense responses to herbivory (Howe and Jander, 2008). Because both genes were induced by JA, we reasoned that they may be part of the wound and/or insect-mediated signal transduction pathways. Indeed, both genes were wound-inducible in leaves with *ZmLOX5* displaying

much higher expression compared to *ZmLOX4*. In agreement with the potential function of *ZmLOX5* in defense against insects, this gene was induced by infestation with fall armyworms. Since *ZmLOX4* was induced by both JA and to a lesser extent by wounding in leaves, it was surprising that the gene was not responsive to insect herbivory. These results indicate that *ZmLOX4* is unlikely to be involved in maize defense against leaf herbivory but may function in other JA-mediated pathways, for example, those induced by pathogen infection. The existence and the molecular switches of separate wound- and pathogen-induced JA-dependent pathways have been reported previously (Lorenzo et al., 2004; Dombrecht et al., 2007).

The unusual responsiveness of *ZmLOX5* expression to mechanostimulation unavoidably associated with treatments of the control plants prompted us to test whether the two genes are induced systemically by mechanical wounding of organs other than leaves. *ZmLOX5* was induced in systemic stems and leaves in response to stem excision or mechanical wounding of roots, but not in the wounded roots. In sharp contrast, *ZmLOX4* was locally induced in roots by wounding but barely detectable in systemic leaves. These findings once again imply that these two closely related paralogs have subfunctionalized in terms of their differential regulation by wounding in above- and under-ground organs. One potential implication from our findings is that *ZmLOX5* can be used as a valuable molecular marker for wound-and insect-triggered defense responses in leaves and root-to-stem wound signal communication. On the other hand, *ZmLOX4* can be a good marker to study wound-mediated responses in maize roots.

Our results point to a possibility that the two genes may have a role in maize interactions with foliar fungal pathogens. Unlike all other treatments, expression of both genes was induced to similar levels during both compatible and incompatible interactions with *C. carbonum*. Interestingly, both genes displayed relatively higher transcript accumulation when the plants were susceptible. The stronger induction of the 9-LOXs in susceptible plants may not be surprising in the light of the important recent discovery that disruption of another maize 9-LOX gene, *ZmLOX3*, resulted in enhanced resistance to several foliar and root-rotting pathogens of maize (Gao et al., 2007; Isakeit et al., 2007), indicating a novel role for some 9-LOXs in facilitating disease development rather than defense.

In summary, we report that duplicated despite their extreme sequence identity 9-LOX paralogs, *ZmLOX4* and *ZmLOX5,* are distinctly involved in maize responses to insect herbivory, wounding, pathogen infection and exogenous application of stress hormones. The differential transcriptional regulation of these 9-LOX paralogs is reminiscent of previously reported differential regulation of another pair of maize segmentaly duplicated 13-LOX paralogs, *ZmLOX10* and *ZmLOX11* (Nemchenko et al., 2006). This study presents yet another example of evolutionary employment of duplicated genes that may have evolved distinct functions, a phenomenon often reported for paralogs as opposed to orthologs. To elucidate the precise physiological functions of *ZmLOX4* and *ZmLOX5*, *Mutator*-transposon insertional mutants of these genes are being generated for further studies.

## MATERIALS AND METHODS

### Plant material and hormonal treatments

Maize seeds of inbred line B73 were grown in a growth chamber and/or a greenhouse in 7 cm diameter pots in Strong-Lite® commercial soil (Universal Mix, Pine Bluff, AZ, USA), temperature was controlled from 22–30 $^{\circ}$C under a 16 h day length, 50% average relative humidity, 560–620 micromole of light from both sun and lamp. For organ-specific expression studies, except for stalk, ear, tassel, silk and leaf, we used seedlings at the V2 developmental stage (second leaf has a visible "collar" at the base of the leaf). We harvested stalk, tassel, ear, silk and leaf tissues from mature plants at the R1 stage (reproductive "milk stage" in kernel development). For analysis of gene expression in underground organs, seeds of B73 inbred line were germinated in the dark using germination papers and grown at room temperature until seedlings reached V2 developmental stage. For plant hormonal treatments, V2 seedlings grown in pots were sprayed with 100 ml of either 0.1% ethanol, 0.01% Tween-20 water solution (control) or 200 μM MeJA, 100 μM ABA (Sigma, St Louis, MO, USA), or 2.5 mM SA. Unlike other hormonal treatments, 10 μl $l^{-1}$ of ethylene was used in hermetically sealed 5.6 L desiccators. For all plants, we harvested at least two seedlings per replicate and three replicates at specific time points after the treatments, frozen immediately in liquid $N_2$, stored at -80 $^{\circ}$C or ground immediately for further extraction of total RNA.

**Mechanical wounding**

For wounding experiments, second leaves of V2 stage seedlings were wounded by crushing the leaf blade with a hemostat perpendicular to the main vein (avoiding damage to the main vein) and the entire wounded leaf was harvested at specific time points. To study systemically induced gene expression in leaves, third leaves of V3 stage seedlings were wounded as described above and the emerging fourth untreated leaves were harvested. To study systemic signaling between roots and leaves (Fig. 6), ten-day-old seedlings were grown on germination paper under light at room temperature and roots were wounded using a hemostat with each wound site approximately 1 cm apart. Both roots and leaves were harvested from either wounded or non-wounded control plants, frozen immediately in liquid $N_2$, stored at -80 $^{\circ}$C or ground immediately for further RNA expression studies. For all experiments, each replicate consisted of at least two seedlings and three replicates were analyzed for each time point.

**Construction of over-expression vectors and analysis of the biochemical properties of ZmLOX5**

To produce recombinant ZmLOX5 protein, over-expression vector containing open reading frame (ORF) of *ZmLOX5* was constructed as described by Nemchenko et al., (2006). The *E.coli* cultures containing the ZmLOX5 expression construct were grown for overnight at 37 $^{\circ}$C in LB broth until the cell culture reached a density value of

$A_{600}$=0.7. Cultured cells of *E.coli* were induced by adding of 1 mM of IPTG to express of the constructs at 15 $^{\circ}$C for 48 h. After centrifugation cells were resuspended in 30 ml lysis buffer (50 mM Tris-HCl, pH 7.5) containing 10% (v/v) glycerol, 0.5 M NaCl, and 0.1% Tween-20 and disrupted by sonication. Cell debris and membranes were removed by centrifugation (12000 g, 15 min). After centrifugation, the supernatant was incubated with linoleic acid as a substrate (120 μM final concentration) in 1 ml of 0.1 M sodium phosphate buffer (pH 5.7-7.0) or 0.1 M Tris buffer (pH 7.7-8.6) for 20 min at room temperature. This reaction was stopped by adding 100 μl of glacial acetic acid. Organic phase was vacuum dried to remove solvent. Remaining lipids were re-suspended in 0.1 ml of high-performance liquid chromatography (HPLC) solvent. The oxidation products were measured by HPLC as described in Nemchenko et al., (2006).

**Quantification of 9-oxylipins in response to wounding**

Leaves of inbred line B73 seedlings were wounded by hemostat as described above and harvested at 0, 4 and 8 h after wounding. 9-oxylipins were measured at the University of North Texas (Denton, TX, USA) as described in Göbel et al., (2002) with some modifications. Five biological replicates were analyzed with each replicate containing leaves from two seedlings. Frozen leaves (0.6-1.0 g) were ground with liquid $N_2$ and extracted by adding 4 ml HIP solution [hexane:isopropanol, 3:2 (v/v) with 0.0025% (w/v) butylated hydroxytoluene]. During extraction process, 500 ng of the internal standard, 13-gamma-HOT (Cayman chemical company, Ann Arbor, MI, USA)

was added. The extract was shaken at 4 °C for 1 h and centrifuged at 2000 rpm for 5 min. The upper phase was transferred into a new tube, mixed with 6 ml of potassium sulphate solution, shaken at 4 °C for 30 min, and centrifuged at 2000 rpm for 5 min. Upper hexane rich layer was transferred into a glass tube, dried under $N_2$, dissolved in 200 µl of hexane:isopropanol [100:5 (v/v)]. Dissolved solvent was dried under $N_2$ and re-dissolved in methanol for reversed phase (RP)-HPLC. 9-oxylipins were purified and quantified on RP-HPLC using a Nucleosil 120-5 C18 column (4.6x150 mm, 5 µm of particle size; Macherey-Nagel, Bethlehem, PA, USA) and two solvent systems [solvent A (methanol:water:acetic acid (80:20:0.1 by vol.) and solvent B (methanol:acetic acid (100:0.1 by vol.)) and a flow rate of 0.18 ml $min^{-1}$. After RP-HPLC normal phase HPLC was carried out on Zorbax Rx-SIL column (2.1x150 mm, 5 µm of particle size, Agilent, Waldbronn, Germany) with a solvent of hexane:isopropanol:trifluoroacetic acid (100:1:0.02 by vol.) and a flow rate of 0.125 ml $min^{-1}$ .

**Isolation of genomic DNA and Southern blot analysis**

For Southern blot analysis, V2 stage seedlings of maize inbred lines B73, Mo17, and A632 were used for extraction of genomic DNA as described by Zhang et al., (2005), and genomic DNA (10 µg) of each inbred line was then digested by five restriction enzymes, *EcoR*I, *BamH*I, *Hind*III, *Xho*I, and *Xba*I, for overnight at 37 °C. Separation of digested DNA was carried in a 0.8% agarose of a TAE-based electrophoresis gel. Separated genomic DNA was transferred with 25 mM phosphate transfer buffer (pH 6.5)

to a nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN, USA) for overnight and then cross-linked to the nylon membrane by a UV cross-linker. The blots were hybridized for overnight at 42 °C with the $^{32}$P-labelled *cpidd40* probe (this probe was generated from a 1.5 Kb EST clone cpidd40 representing 3' portion of the *ZmLOX4* gene) in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA), and washed according to the manufacturer's instruction. The cpidd40 probe was generated using PCR with the following primers LOX40f-*Bam*HI, 5□'-TGG GAT CCA TGG CGA GCC TGA GCG CTG GAC G-3'; LOX40r-*Nsi*I, 5'-CAA TGC ATA TCG AGA CGT GGC TCC TCT CT-3'. The membrane was exposed to a BioMax X-ray film (Kodak, Rochester, NY, USA) in a cassette for seven days.

**Total RNA extraction and Northern blot analysis**

Total RNA from diverse maize tissues was extracted as described in Nemchenko at al., (2006) by TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocols. Ten to 15 μg of RNA was separated in 1.5% (w/v) formaldehyde agarose gel in 1X MOPS buffer and was transferred onto a nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN, USA). Equal loading of RNA in the gels RNA samples was verified by gel staining with ethidium bromide and exposure to UV light. Membranes were hybridized with 50 to 100 ng of $^{32}$P-labelled gene-specific probes in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) at 45 °C for overnight. Washes were performed twice with 2X SSC,

0.1% SDS for 15 min at 65 °C and twice with 0.1X SSC, 0.1% SDS for 20 min at 65 °C. Membranes were then dried at room temperature for 30 to 40 min and were exposed to a BioMax X-ray film (Kodak, Rochester, NY, USA) in a cassette. All experiments were repeated at least twice. The following primers were used to generate gene-specific probes: for the *ZmLOX4* gene; LOX4 forward, 5'-ATC TCC ATA TGA GCC TCC TCA CTG CC-3'; LOX4 reverse, 5'-CAC ACA TGA CAA CAT TAT CCA GAC G-3'; for the *ZmLOX5* gene; LOX5 forward, 5'-CTC CAT ATG AGC CTG GGC AGA TTG-3'; LOX5 reverse, 5'-CAA GCG TGG ACT CCT CTC TC-3'.

**Dot blot assay**

Dot blot assay was performed to confirm gene-specificity of the probes used for all northern blot analyses in the study. For this, plasmid DNA containing cDNAs of either the *ZmLOX4* or *ZmLOX5* genes was blotted onto nylon membranes as described in Nemchenko et al., (2006), and the membranes were hybridized with the $^{32}$P-labeled gene-specific probes (generated by PCR using the primers listed above) in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA). Membranes were hybridized and washed as described above for northern blot analyses. Membranes were exposed to BioMax X-ray films for 36 h at -80$^{\circ}$C.

**Inoculation with *Cochliobolus carbonum***

Two-week-old maize seedlings of Pr inbred line, which is susceptible to *Cochliobolus carbonum* race 1 tox2+ (virulent wild type, HC-toxin-producing strain) but is resistant to *C. carbonum* race 1 tox2- (avirulent mutant strain, deficient in HC-toxin production) (Multani et al., 1998), were infected as described in Nemchenko et al., (2006). For inoculations, we used conidial suspensions containing $10^5$ conidia per ml in 0.1% Tween-20 solution. Control plants were treated with sterile water containing 0.1% Tween-20 as mock treatment. Seedling plants were inoculated by spraying the leaves to imminent run-off with conidial suspension. After inoculation, seedlings were immediately covered with plastic bags to maintain high humidity and were incubated for 3, 6, 12, 24, 48, or 72 h under typical greenhouse conditions as described above. After each incubation time, infected leaves were harvested and frozen quickly in liquid $N_2$ and kept at -80 $^{\circ}$C until used for extraction of total RNA.

**Infestation by fall armyworm**

Maize plants (inbred line B73) were grown until V6 stage under greenhouse conditions as described above, and were infested with 0 (control) or 10 neonates (newly-hatched) of the fall armyworm [*Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)] at the whorls of the plants. The infested or non-infested (controls) maize leaves of similar age were harvested at 0, 1, 4, and 8 d after the infestation. Six plants

(two biological replicates) were used for each insect treatment at each sampling date. All leaf samples were flash-frozen and grinded in liquid $N_2$ and stored at -80 $^{\circ}$C until used for RNA extraction.

# CHAPTER III

## MAIZE 9-LIPOXYGENASE GENE, *ZMLOX5*, IS INVOLVED IN THE REGULATION OF WOUND-INDUCED JASMONIC ACID BIOSYNTHESIS AND RESISTANCE TO INSECT HERBIVORY

## INTRODUCTION

Like animals, plants activate defensive mechanisms when attacked by insects and pathogens. One of these induced defense responses is enzymatic and non-enzymatic lipid peroxidation, which has been shown to play important roles in plant development and survival under a variety of environmental stresses. One largely understudied group, consisting of an estimated 400 molecular species (Dr. Ivo Feussner, personal communication), resulting from enzymatic lipid peroxidation processes are oxylipins, a collective name for oxygenated polyunsaturated fatty derivatives of α-dioxygenase and lipoxygenase (LOX) reactions.

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This chapter is in preparation for journal submission. Contributing researchers that will be listed as authors on the publication are: Yong-Soon Park, Xiquan Gao, Jantana Keereetaweep, Aruna Kilaru, Shawn A. Christensen, Robert Meeley, Kent D. Chapman, Stefan Vidal, and Michael V. Kolomiets. All the experiments were carried out by myself with the exception of oxylipin profiling (assisted by Jantana Keereetaweep, Aruna Kilaru and Kent D. Chapman ), JA analysis (assisted by Shawn A. Christensen) and beet armyworm assays (assisted by Stefan Vidal).

In plants, LOX pathways begin when molecular oxygen is incorporated into either the 9- (9-LOX) or the 13-position (13-LOX) of the C18 carbon chain of polyunsaturated fatty acid (PUFAs), such as linoleic (C18:2) and linolenic (C18:3) acid (Feussner and Wasternack, 2002). The best understood, in terms of physiological functions, are the 13-LOX derived oxylipins jasmonic acid and its derivatives (JAs) and green leaf volatiles (GLV) (Koch et al., 1999; Kessler and Baldwin, 2002). JAs and GLVs have been demonstrated to regulate diverse plant defenses to a number of stressors (Vancanneyt et al., 2001; Nakamura and Hatanaka, 2002; Glazebook, 2005; Matsui, 2006; Wasternack, 2007; Baldi and Devoto, 2008; Howe and Jander, 2008). Our knowledge of the biological relevance of a large group of 9-LOX-derived metabolites and signal cascades regulated by them is still fragmented.

Similar to the 13-LOX pathway, 9-LOX primary metabolites, 9-hydoperoxides, are further metabolized by a number of enzymes, including peroxygenase (POX), hydroperoxide lyase (HPL), divinyl ether synthase (DES), reductase, epoxy alcohol synthase (EAS) and LOX itself (Fig. 8). The POX and reductase pathway branches convert 9-hydroperoxides (9-HPODE/TE) to 9-hydroxides (9-HODE/TE) (Feussner et al., 1998). HPL produces 9-oxylipin derived GLVs, aldehydes (C6 or C9) and the corresponding fatty acids due to the oxidative cleavage of the hydrocarbon backbone of 9-hydroperoxides (Matsui, 1998). In Solanaceous species, colneleic acid (CA) and colnelenic acid (CnA) are formed by 9-DES (Grechkin, 1998).

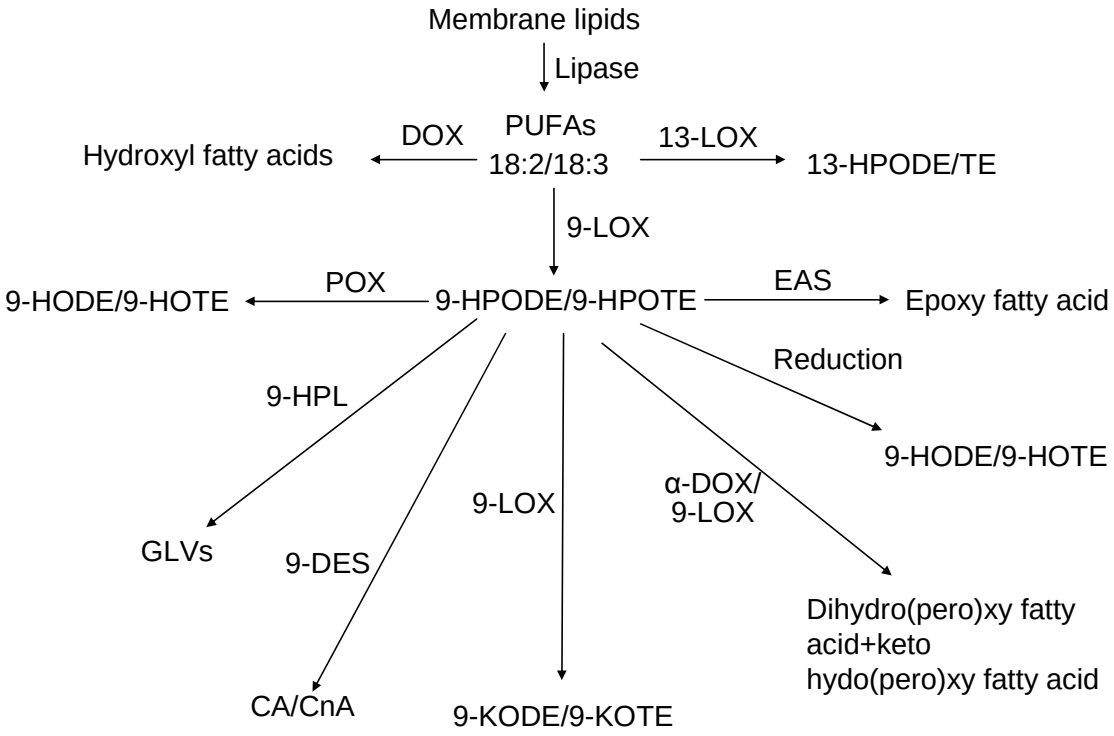


**Figure 8.** Schematic representation of plant 9-lipoxygenase (LOX) pathway. Hydroperoxides derived from 18:2/18:3 by either 9- or 13-LOXs are metabolized by several LOX pathway branches to form diverse oxylipin compounds. 18:2, linoleic acid; 18:3, linolenic acid; CA, colneleic acid; CnA, colnelenic acid; GLVs, green leaf volatiles. Linoleic acid-derived oxylipins: 9*S*-HPODE, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid; 9*S*-HODE, (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid; 9-KODE, keto(10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid; 13*S*-HPODE, (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid; 13*S*-HODE, (9*Z*,11*E*,13*S*)-13-hydroxy-9,11-octadecadienoic acid; 13-KODE, keto(9Z,11E)-13-hydroxy-9,11-octadecadienoic acid; (12,13*S*)-EODE, (12,13*S*)-epoxy. Linolenic acid-derived oxylipins: 9*S*-HPOTE, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecadienoic acid; 9*S*-HOTE, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid; 9-KOTE, keto(10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid. 13*S*-HPOTE, (9*Z*,11*E*,13*S*,15*Z*)-9-hydroperoxy-9,11,15-octadecatrienoic acid; 13*S*-HOTE, (9*Z*,11*E*,13*S*,15*Z*)-9-hydroxy-9,11,15-octadecatrienoic acid; 13-KOTE, keto (9*Z*,11*E*,15*Z*)-9-hydroxy-9,11,15-octadecatrienoic acid; 12-OPDA, 12-oxo-phytodienoic acid. Enzymes responsible for formation of oxylipins: DOX, dioxygenase; DES, divinyl ether synthase; EAS, epoxy alcohol synthase; HPL, hydroperoxide lyase; LOX, lipoxygenase; POX, peroxygenase.

LOX itself converts 9-HPODE/TE to 9-ketones such as 9-KODE/TE under low oxygen conditions (K□hn et al., 1990). Epoxy fatty acids are formed by EAS due to the intramolecular rearrangement of the 9-hydroperoxides (Hamberg, 1999).

Thus far, most published evidence implicates 9-LOXs in plant interactions with pathogens (Weber et al., 1999; Göbel et al., 2001, 2002; Ponce de Leon et al., 2002; Hamberg et al., 2003; Gao et al., 2007, 2009), with only a few studies suggesting a role in growth and development (Kolomiets et al., 2001; Vellosillo et al., 2007). Specifically, down-regulation of a pathogen inducible 9-LOX in tobacco resulted in increased susceptibility to *Phytophthora parasitica* (Rance et al., 1998), whereas overexpression enhanced resistance (Mene-Saffrane et al., 2003). Hypersensitive response (HR) reactions correlated with 9-LOX reactions in tobacco (Cacas et al., 2005, 2009), and 9-oxylipin insensitive mutant in Arabidopsis displayed enhanced susceptibility to a bacterial pathogen (Vellosillo et al., 2007).

Relative to plant pathogen interactions studies, only a few studies have implicated 9-LOX pathway metabolism in plants interactions with insects. Several 9-oxylipins were upregulated in potato after infestation of aphid (Gosset et al., 2009). Down regulation of the rice OsLOX1, which displayed both 13- and 9-LOX activity in vitro, led to increased susceptibility to brown plant hopper attack (Wang et al., 2008). Recently, we showed that one of the two closely related duplicated genes of maize, *ZmLOX5,* was upregulated in leaves by treatments with JA, wounding and fall armyworm feeding (Park et al., 2010). To test the hypothesis that *ZmLOX5* is specifically involved in host defense against insect herbivory, we have created three

independent *Mutator* transposon insertional knock-out alleles of *ZmLOX5*. Disruption of *ZmLOX5* resulted in moderate but significant reduction of several 9-and 13-oxylipins in wounded leaves. Surprisingly, *lox5* mutants showed no increase in JA levels in response to wounding compared to wounded WT, which was accompanied by altered expression of putative JA-producing 13-LOX. The *lox5* mutant showed increased susceptibility to insects both under laboratory and field conditions. Overall, this study indicates that a maize 9-LOX, *ZmLOX5*, is required for normal levels of resistance to chewing insect herbivores, and provides genetic evidence for specific 9-LOX pathway requirement for wound-induced JA biosynthesis.

## RESULTS

### The *lox5* mutant alleles are strong loss-of-function alleles of *ZmLOX5*

A reverse genetic *Mutator* transposon tagging approach (Meeley and Brings, 1995) was used to generate the knock out mutant alleles of *ZmLOX5* to elucidate physiological functions of this gene. The PCR-based screening using a *Mu*-specific primer and several *ZmLOX5*-specific primers (Table 3) of approximately 42,000 mutant individuals yielded three independent *Mu*-insertional alleles of *ZmLOX5*. Sequencing of the regions flanking *Mutator* insertion sites revealed that *lox5-1* allele had a *Mu* insertion in exon 2 and *lox5-2* and *lox5-3* alleles contained insertions in exon 9 (Fig. 9A).

**Table III**. Primer sequences of tested genes in this study

| Gene name | Primer (5'-3') |
|---|---|
| *ZmLOX1-F* | TCTGTCTGAGCTGAGGACGTA |
| *ZmLOX1-R* | CACAAAGTAACTTCATTATTGAGGA |
| *ZmLOX2-F* | TTCCATCTGATTCGATCGAG |
| *ZmLOX2-R* | CACATTATTATTGGGAAACCAAC |
| *ZmLOX4-F* | ATCTCCATATGAGCCTCCTCACTGCC |
| *ZmLOX4-R* | CACACATGACAACATTATCCAGACG |
| *ZmLOX5-F* | CTCCATATGAGCCTGGGCAGATTG |
| *ZmLOX5-R* | CAAGCGTGGACTCCTCTCTC |
| *ZmLOX10-F* | ATCCTCAGCATGCATTAGTCC |
| *ZmLOX10-R* | AGTCTCAAACGTGCCTCTTGT |
| *ZmACO31-F* | GTTCCCGTGATCGACTTCTC |
| *ZmACO31-R* | GAAGATGTCCTCCCAGTCCA |
| *GAPDH-F* | GCTAGCTGCACCACAAACTGC |
| *GAPDH-R* | TAGCCCCACTCGTTGTCGTAC |
| 9242 | AGAGAAGCCAACGCCAWCGCCTCYA |
| *lox5-1F* | GAAAAGCGAAGAGCGGCCATGTT |
| *lox5-1R* | TTCTTGGCTGCCACCGAGGATC |
| *lox5-2F* | GCGGTGATCGAGCCGTTCGTAATC |
| *lox5-2R* | CGCGGATGCCACCCAGATGAT |
| *lox5-3F* | TGCCGGACCAGTCAAGCCCATAT |
| *lox5-3R* | GGCCCCTTCCGGTTCTTCAAGTC |

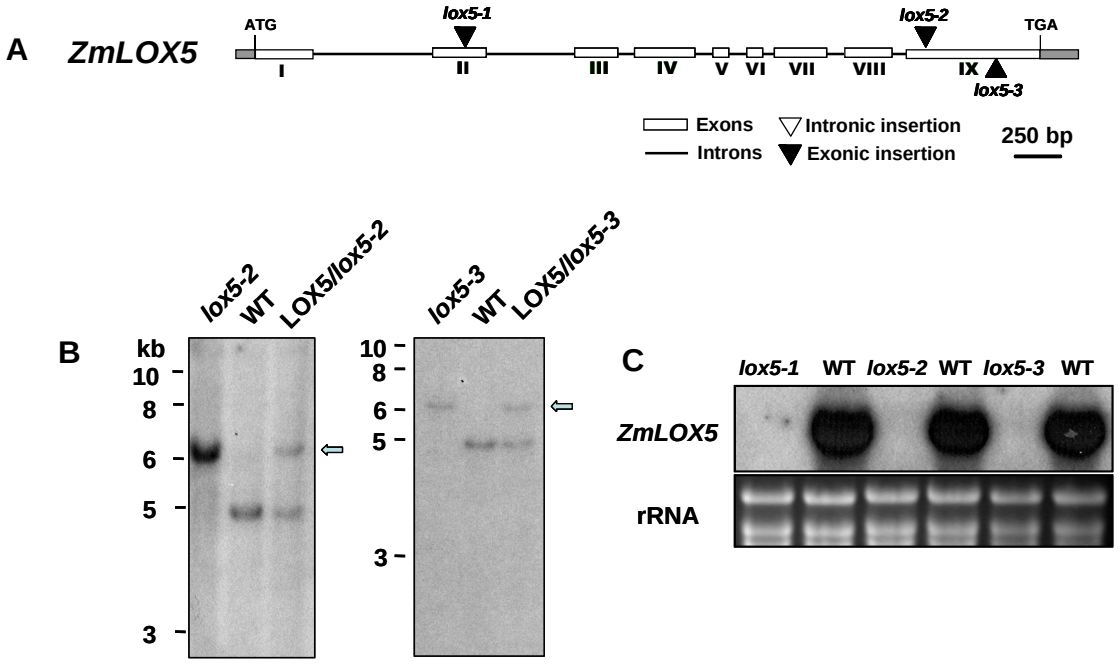


**Figure 9**. The *lox5* mutant alleles are strong loss-of-function alleles of the *ZmLOX5* gene.

A, Schematic representation of the genomic structure of the *ZmLOX5* indicating the *Mutator* element insertional sites (shown as triangles). B, Southern blot analysis showing restriction size polymorphism in due to insertion of *Mu*-elements. Genomic DNA (15 µg per lane) digested with *Eco*RV was separated in an agarose gel and hybridized with *ZmLOX5* gene-specific probe. The arrows represent the mutant band. WT, wild-type near-isogenic siblings; *lox5-2* and *lox5-3*, homozygous mutants; LOX5/*lox5-2* and LOX5/*lox5-3*, heterozygous individuals. C, Northern blot analysis of the *ZmLOX5* transcripts in shoot apical meristem of the *lox5* mutant alleles and corresponding near-isogenic wild type.

To remove transposable elements inserted elsewhere in the genomes of the original mutant individuals, these individuals were back crossed into B73 inbred line seven times ($BC_7$) yielding near-isogenic lines (NILs) wild type and mutant lines at the *ZmLOX5* locus.

Southern blot analysis shows that *ZmLOX5* gene-specific probe (Park et el., 2010) hybridized to a ~4.9 Kb restriction fragment of wild type DNA digested with *Eco*RV, while *lox5-2* and *lox5-3* homozygous lines displayed a fragment that was about 1.1 kb longer than the wild type while heterozygous individuals produced both the mutant and wild type fragments (Fig. 9B), thus confirming the presence of *Mu* insertions in the gene. Because *ZmLOX5* was highly expressed in unchallenged shoot apical meristem (SAM) (Park et al., 2010), we compared steady-state transcript accumulation of the *ZmLOX5* gene in SAM of wild-type (WT) and the mutants. A northern blot assay showed that transcripts of *ZmLOX5* could not be detected in all three alleles (Fig. 9C), indicating that they are loss-of-function mutations.

**_lox5_ mutants are more susceptible to insect herbivory**

In our previous study, *ZmLOX5* transcripts were shown to be dramatically induced by insect feeding, which contrasted with the lack of detectable induction of the duplicate *ZmLOX4* gene (Park et al., 2010). Therefore, we hypothesized that *ZmLOX5* may be specifically involved in maize defense to insect herbivory.

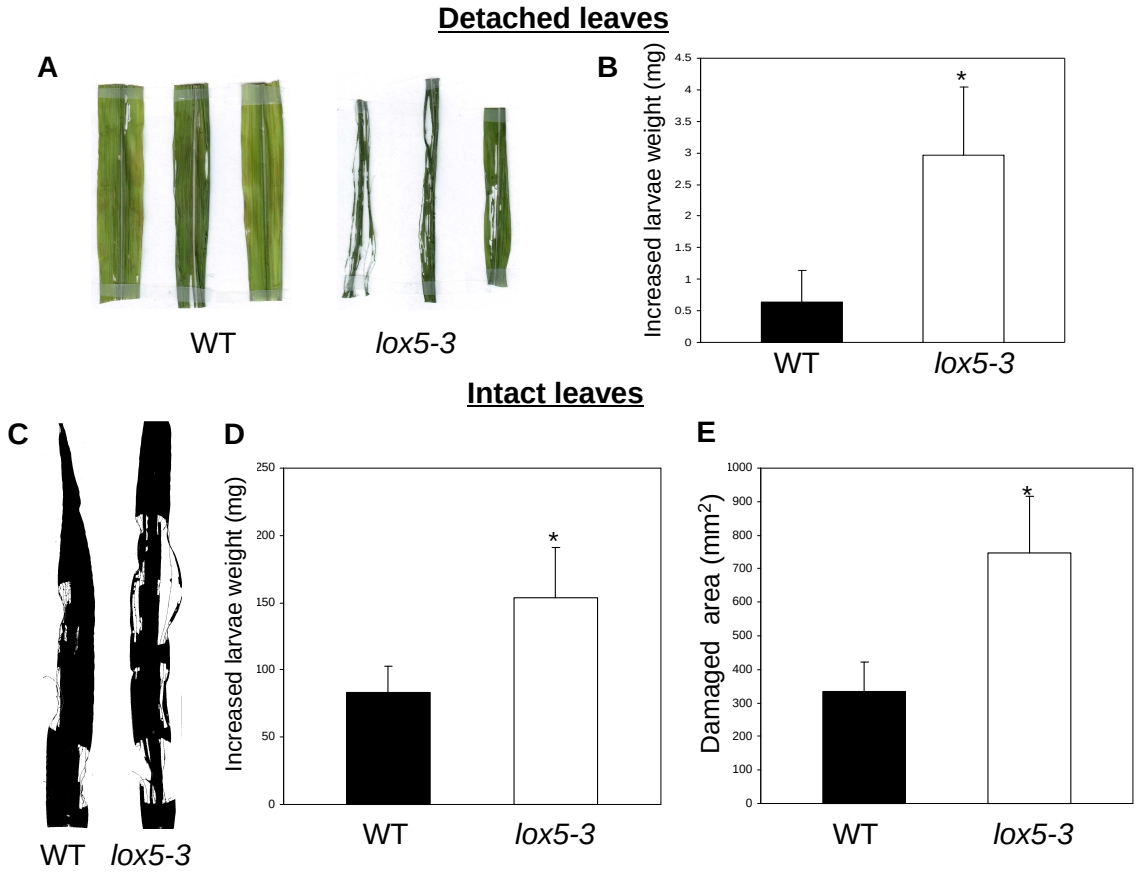


**Figure 10**. Infestation of beet armyworm (BAW, *Spodoptera exigua*) in detached and intact leaves.

A, Insect performance bioassay in detached leaves. Third instar *Spodoptera exigua* larvae were infested on detached leaves of wild type (WT) and *lox5-3* mutant and were allowed to feed for two days before pictures were taken. B, Two days post-infestation, larvae were collected and weighed. Five replicates were tested for these experiments. C, Insect performance bioassay in intact leaves. Second to third instar *Spodoptera exigua* larvae were placed on young seedling leaves and were allowed to feed for three days. Larvae were weighed (D) and infested area measured using the ImageJ software three days post-infestation (E). Seven replicates were used for the results shown in C to E,. Asterisks indicate significant differences between WT and *lox5-3* mutant using SPSS program ($P<0.05$).

To test this hypothesis, WT and *lox5* mutant NILs were compared for resistance to infestation by beet armyworm (BAW), *Spodoptera exigua*, by monitoring both leaf area eaten by larvae and insect performance as measured by gain in weight. Beet armyworm feeding resulted in visibly greater leaf damage that was observed in both detached (Fig. 10A) and intact leaves (Fig. 10C). Increased area was accompanied by increased weight gain of larvae fed on *lox5* mutant in both feeding trials (Fig. 10B, 10D and 10E).

Insect damage to the mutant and WT NILs were also recorded under natural field infestation conditions for several years. Consistently greater insect damage was observed in B73 genetic background for all three mutant alleles. Similarly, disruption of the *ZmLOX5* gene in two other genetic backgrounds, W438 and CML, resulted in increased susceptibility to insect herbivory under natural infestation conditions found in the field both 2009 and 2010 growing seasons (Fig. 11A and 11B). In addition, greater leaf susceptibility to insects was accompanied by greater insect damage to the cobs recorded for all genetic backgrounds tested (Fig. 12). Taken together, these data strongly implicate *ZmLOX5*-mediated oxylipin metabolism in maize defense against insect herbivory.

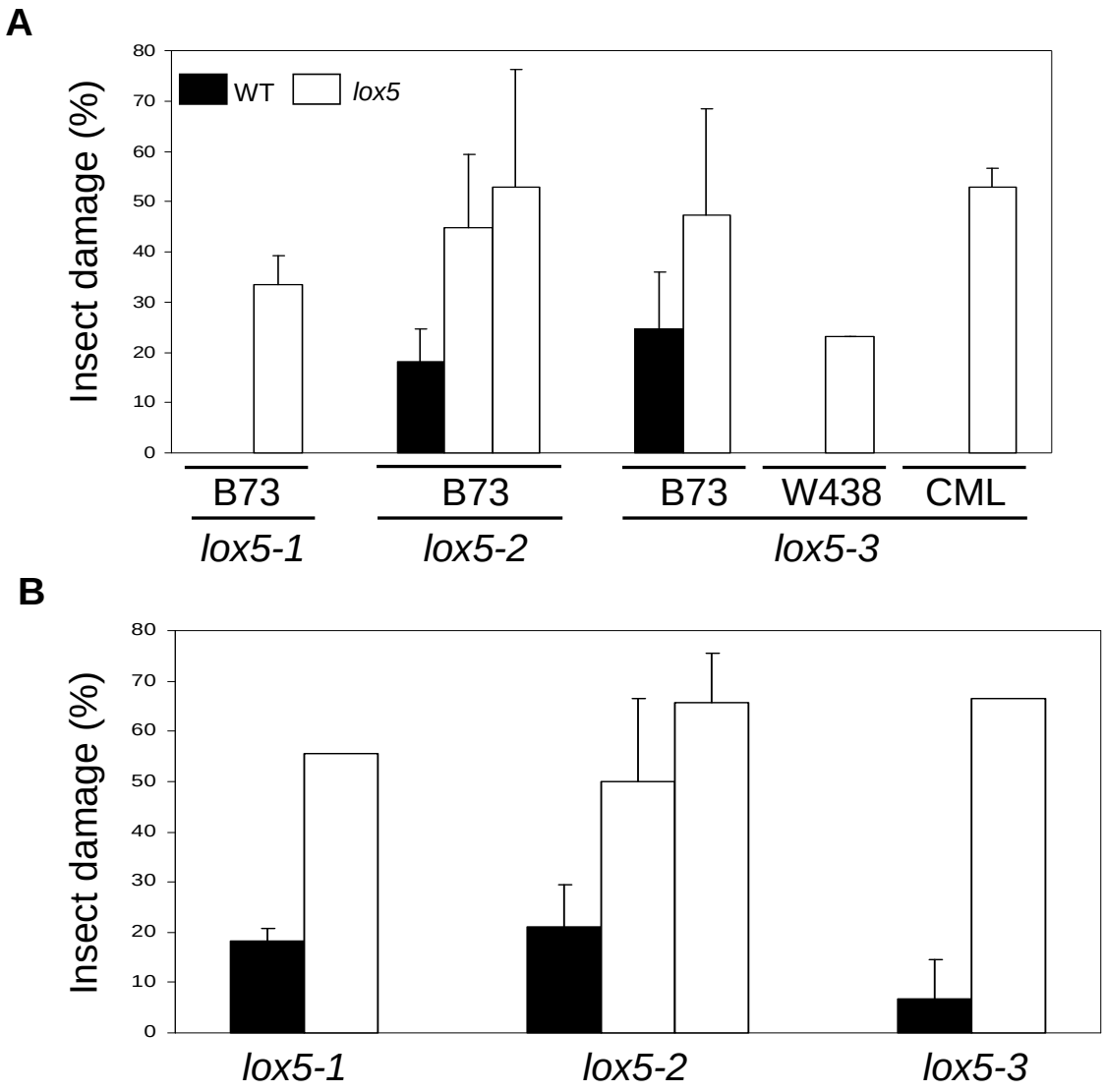


**Figure 11**. Insect performance in leaves of wild-type (WT, black bar) and *lox5* mutants (White bar) under field natural infestation conditions. Insect susceptibility was quantified in leaves of all three mutant alleles and near-isogenic WT in College Station, TX in 2009 (A) and 2010 (B).

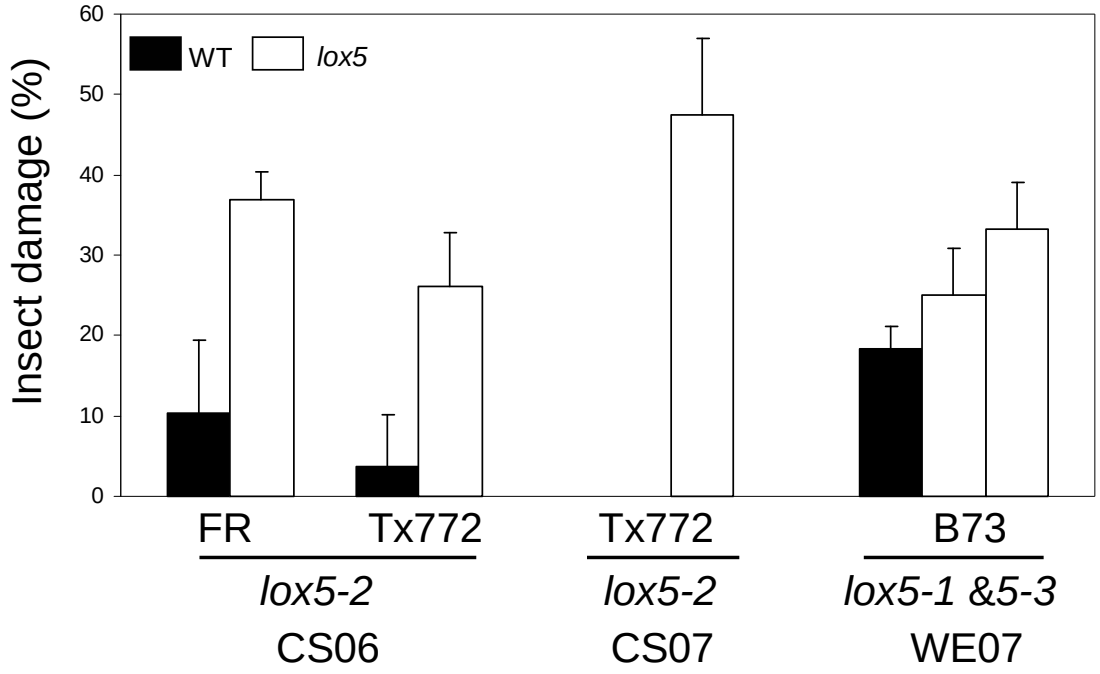


**Figure 12**. Insect performance in cobs of wild-type (WT, black bar) and *lox5* mutants (White bar) under field natural infestation conditions. Insect susceptibility was quantified in leaves of all three alleles and mutant and near-isogenic WT in College Station and Weslaco, TX.

**Levels of 9- and 13-oxylipins are reduced in *lox5* mutant**

Since *ZmLOX5* was strongly induced in leaves by wounding (Park et al., 2010), we tested whether disruption of *ZmLOX5* led to reduced biosynthesis of 9-and 13-oxylipins in response to mechanical damage. For this analysis, V4 stage seedlings in the B73 background were wounded, and a subset of well characterized 9- and 13-oxylipins were quantified by using a high-performance liquid chromatography (HPLC). The oxylipins quantified included 9-oxylipins, produced from linoleic acid, 9-hydro(pero)xy-octadecadienoic acid (9-H(P)ODE) and linolenic acid-derived 9-hydro(pero)xy-octadecatrienoic acid (9-H(P)OTE). Among the 13-LOX-metabolized oxylipins, we examined 13-hydro(pero)xy-octadecadienoic acid (13-H(P)ODE), and 13-hydro(pero)xy-octadecatrienoic acid (13-H(P)OTE) (Fig. 8).

In non-wounded leaves, no difference between WT and *lox5* mutants was detected in the accumulation of 9-oxylipins (Fig. 13A). However, 4 h after wounding, content of 9-HOTE ($p=0.017$) and 9-HPOTE ($p=0.042$) were moderately but significantly greater in WT compared to the mutant (Fig. 13A). In addition to induction of several 9-oxylins, higher levels of 13-products were detected in both WT and mutant at 4 h and 8 h after wounding as compared to the unchallenged control leaves. Unexpectedly, *lox5-3* mutant produced statistically lower levels of 13-oxylipins, 13-HPODE ($p=0.047$), 13-HOTE ($p=0.041$), and 13-HPOTE ($p=0.037$), at 8 h after treatment (Fig. 13B).

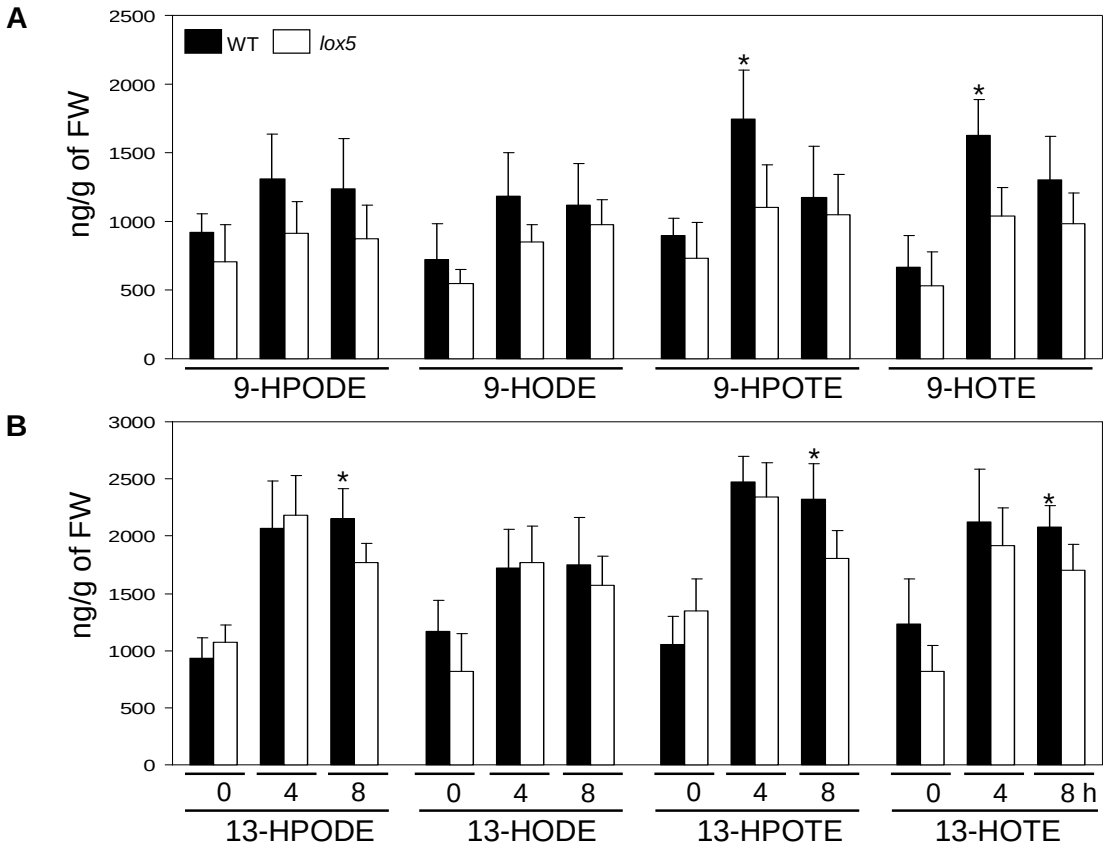


**Figure 13**. Content of C18:2 and C18:3 derived oxylipins extracted from wounded leaves in wild-type (WT, black) and *lox5-3* mutant (white) at 0, 4 and 8 h post treatment.

A, 9-HPODE, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid; 9*S*-HODE, (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid; 9-HPOTE, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecadienoic acid; 9-HOTE, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid. B, 13-HPODE, (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid; 13-HODE, (9*Z*,11*E*,13*S*)-13-hydroxy-9,11-octadecadienoic acid 13-HPOTE, (9*Z*,11*E*,13*S*,15*Z*)-9-hydroperoxy-9,11,15-octadecatrienoic acid; 13-HOTE, (9*Z*,11*E*,13*S*,15*Z*)-9-hydroxy-9,11,15-octadecatrienoic acid. Asterisks indicate significant differences between WT and *lox5-3* mutant using a SPSS program ($P<0.05$).

**Loss of *ZmLOX5* is compensated by increased expression of other LOX genes**

In addition to wound-inducible *ZmLOX1*, which possesses both 9- and 13-LOX activity (Kim et al., 2003), *ZmLOX5* is the major wound-inducible 9-LOX gene in maize (Park et al., 2010). Since ZmLOX5 recombinant protein had a clear 9-LOX regio-specificity (Park et al., 2010), it was surprising that disruption of this isoform resulted only in a relatively small reduction of wound-induced 9-oxylipins (Fig. 13A), suggesting that other 9-LOXs may compensate for the lack of the functional *ZmLOX5* isoform. Indeed, transcripts of 9/13-LOXs, *ZmLOX1* and *ZmLOX2*, as well as that of the closest relative of *ZmLOX5*, *ZmLOX4*, displayed relatively stronger wound induction in the *lox5* mutant as compared to WT (Fig. 14). The wound-inducible pattern of another 9-LOX gene, *ZmLOX3*, was not altered in the *lox5-3* mutant (data not shown). Taken together, our data suggest that other 9- and 9/13-LOXs may compensate for the lack of *ZmLOX5* and produce 9-oxylipins in *lox5* mutant after wounding at the levels close to those observed in WT (Fig. 14).

***ZmLOX5* disruption leads to reduced wound-induced JA but increased ethylene**

Because LOX primary products, 9/13S-HPODE/TE and 9/13S-HODE/TE, were only moderately decreased in the *lox5* mutants (Fig. 13), this decrease alone is unlikely to explain the dramatically increased susceptibility to insect herbivory.

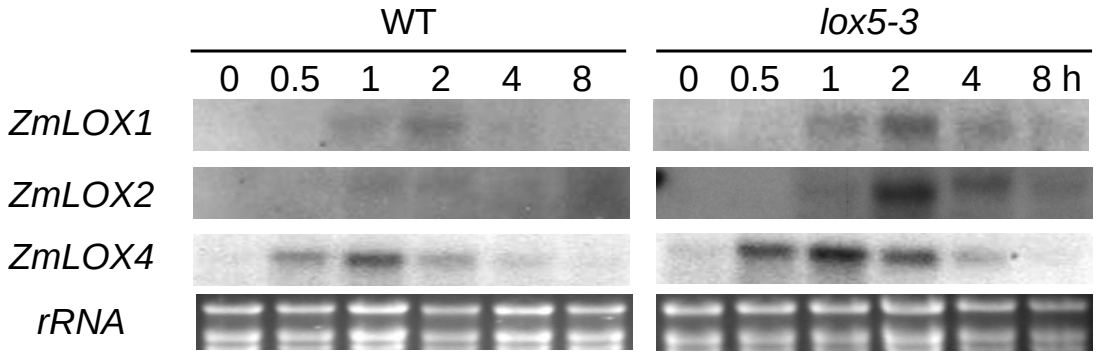


**Figure 14**. Expression of maize 9- and 9/13-lipoxygenase genes in wild-type (WT) and *lox5-3* mutant in response to wounding. Total RNA was extracted from control or wounded WT and *lox5-3* mutant at specified time points (h). Fifteen μg of total RNA was loaded into each lane of 1.5% formaldehyde RNA gel and separated by electrophoresis, transferred onto nylon membranes, and hybridized with $P^{32}$-labeled *ZmLOX5* gene-specific probe. Ethidium bromide staining of gels confirmed equal loading of RNA samples.

Therefore, we tested whether production of JA, the major defense hormone against insect pests, is altered in the mutant at 0, 1, 4 and 8 h after wounding. Although trans-JA was induced in both WT and the *lox5* mutant at 4 and 8 h after mechanical damage, its levels were very low and no significant induction was detected between genotypes (Fig. 15A). However, the levels of cis-JA and total JA were significantly reduced at 1 and 8 h (Fig. 15B and 15C). These data suggest that functional *ZmLOX5* is required for normal wound induction of JA. In agreement with reduced wound-induced JA levels, expression of a 13-LOX gene, *ZmLOX10* (Nemchenko et al., 2006), was significantly decreased in response to wounding in *lox5-3* mutant as compared to WT (Fig. 15D), suggesting that *ZmLOX5* may exert its action on wound-induced JA accumulation through the regulation of the *ZmLOX10* gene.

The JA signaling pathway has been often reported to interact synergistically with ethylene (ET)-mediated signal transduction pathways especially to induce stronger defense response against insect pests (Schmelz et al., 2003; van Loon et al., 2006). Therefore, we measured ET emission is altered in *lox5* mutant in response to wounding. Unexpectedly, the *lox5* mutant emitted higher levels of ET at 1 to 4 h after wounding (Fig. 16A). Greater production of ET may, at least in part, be explained by increased expression ET biosynthesis gene, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase 31 (*ZmACO31*) (Fig. 16B).

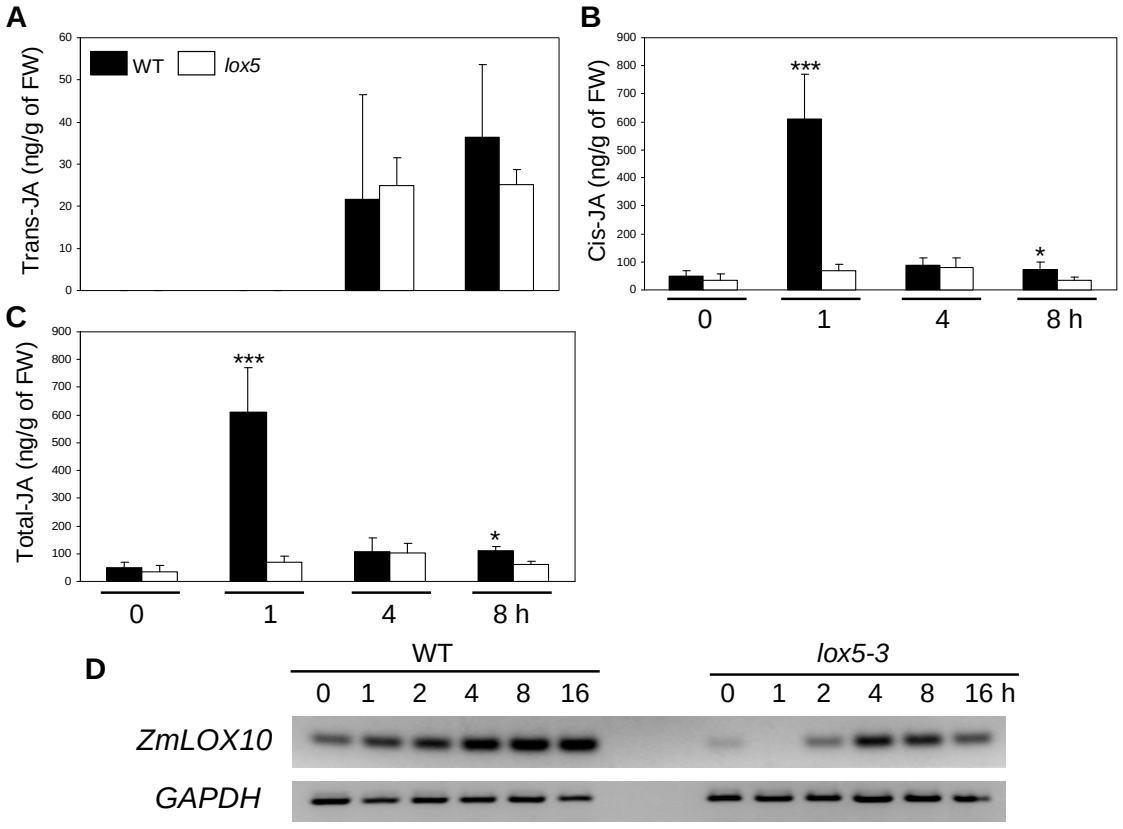


**Figure 15**. Wound-induced jasmonic acid (JA) and expression of putative JA biosynthesis gene are reduced in wounded *lox5* mutant. Accumulation of trans-JA (A), cis-JA (B) or total JA (C) was measured in wild-type (WT) and *lox5-3* mutant by using liquid chromatograshy/mass spectrometry (LC/MS) in response to wounding at different time points.. WT, black bars; *lox5-3* mutant, white bars. D, Expression of a 13-LOX gene, *ZmLOX10*, in WT and *lox5-3* mutant leaves in response to wounding. Transcript levels were measured by semi-quantitative reverse-transcription PCR. Expression of glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was used as a house-keeping control. Asterisks indicate significant differences between WT and *lox5-3* mutant (*** $P<0.001$); * $P<0.05$).

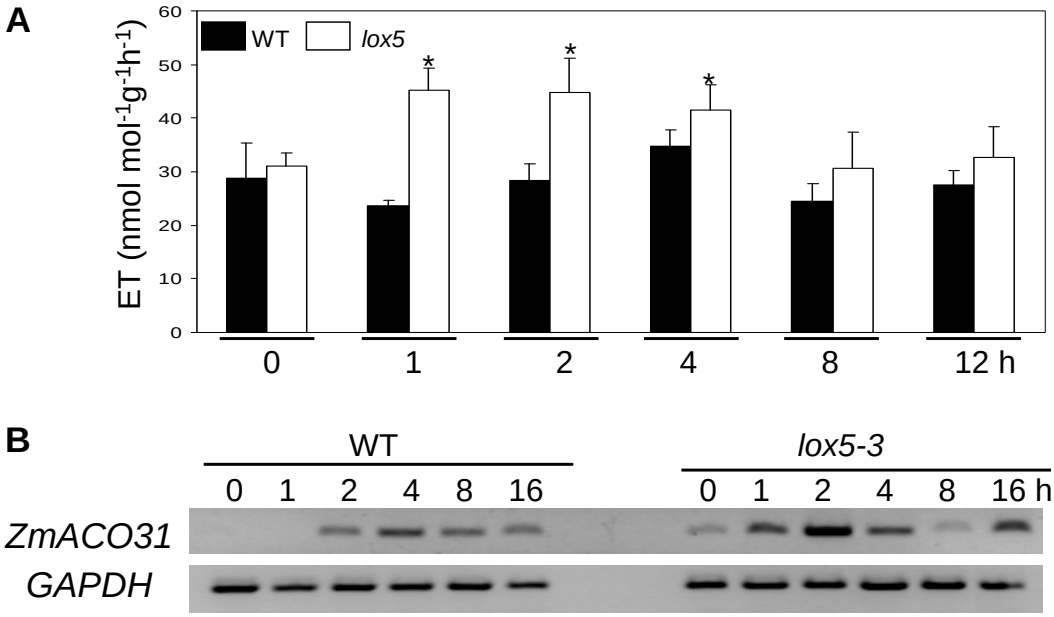


**Figure 16**. Ethylene (ET) emission levels and expression of ET biosynthesis gene *ZmACO31* in wounded wild-type (WT, black bars) and *lox5-3* mutant (white bars). A, ET levels were measured by gas-chromarography as described by Gao et al., (2008). The fourth leaves of V4 stage of seedlings were wounded. B, *ZmACO31* expression in wounded leaves in WT and *lox5-3* mutant was analyzed by by semi-quantitative reverse-transcription PCR. Expression of glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) was used as a standard. Asterisks indicate significant differences between WT and *lox5-3* mutant ( $P<0.05$).

**_ZmLOX5_ is expressed in response to beet armyworm elicitor and ROS-generating microbial elicitors**

In our previous study we showed that, unlike its closest duplicated paralog, *ZmLOX4*, *ZmLOX5* transcripts were strongly induced in leaves by all three treatments typically associated with insect herbivory, JA, mechanical wounding and fall armyworm infestation (Park et al., 2010). In addition, we have noticed that *ZmLOX5* transcripts accumulated in most control, water-treated samples or in seedlings that were transferred to the laboratory bench from another location. These results suggested that this gene is mechanosensitive. To test this hypothesis, induced expression of the *ZmLOX5* was measured in leaves in response to touch stimulation by gentle bending and rubbing seedling leaves. *ZmLOX5* transcripts gradually increased between 1 h and 4 h peaking at 2 h after touch stimulus and declined thereafter (Fig. 17B). Since similar response of *ZmLOX5* was observed in water-sprayed leaves (Park et al., 2010), we tested whether this touch-inducible response of *ZmLOX5* translates into detectable physiological responses to water treatment. We consistently observed visibly greater accumulation of $H_2O_2$ in mutant leaves treated with water drops as compared to similarly treated wild type. Similar results were observed in the control samples in the course of measuring accumulation of $H_2O_2$ in other diverse physiological assays including treatments of ROS-inducing microbial elicitors such as alamethicin and coronitine (Aidemark et al., 2010) (Fig. 17C). These results suggest that *ZmLOX5*-mediated lipid metabolism may

function in repressing uncontrolled ROS accumulation in response to touch stimulus such as wind and rain that plants are constantly exposed to.

To test whether *ZmLOX5* responds to the application of insect elicitors as has been shown for other insect-inducible genes (Erb et al., 2009), the epidermal cell layer of the wild type leaves was scratched with a razor blade (referred to as mild wounding) followed by the application of beet armyworm (BAW) oral secretion (OS). Similarly wounded control plants were treated with the same amount of buffer. *ZmLOX5* transcript accumulation was measured in three different parts of leaves, local wound- or OS-treated tissues, distal tissues (upward area from the treatment site), or systemic leaf. Transcript of *ZmLOX5* was detected at 1 h to 4 h after mild wounding locally and distally (Fig. 17A, top). *ZmLOX5* expression was highly induced at 4 h post when leaves were treated with elicitor, especially in distal leaves (Fig. 17A, bottom) and interestingly, no induction of *ZmLOX5* was detected in systemic leaves by mild wounding or elicitor treatment at the time points tested (Fig. 17A).

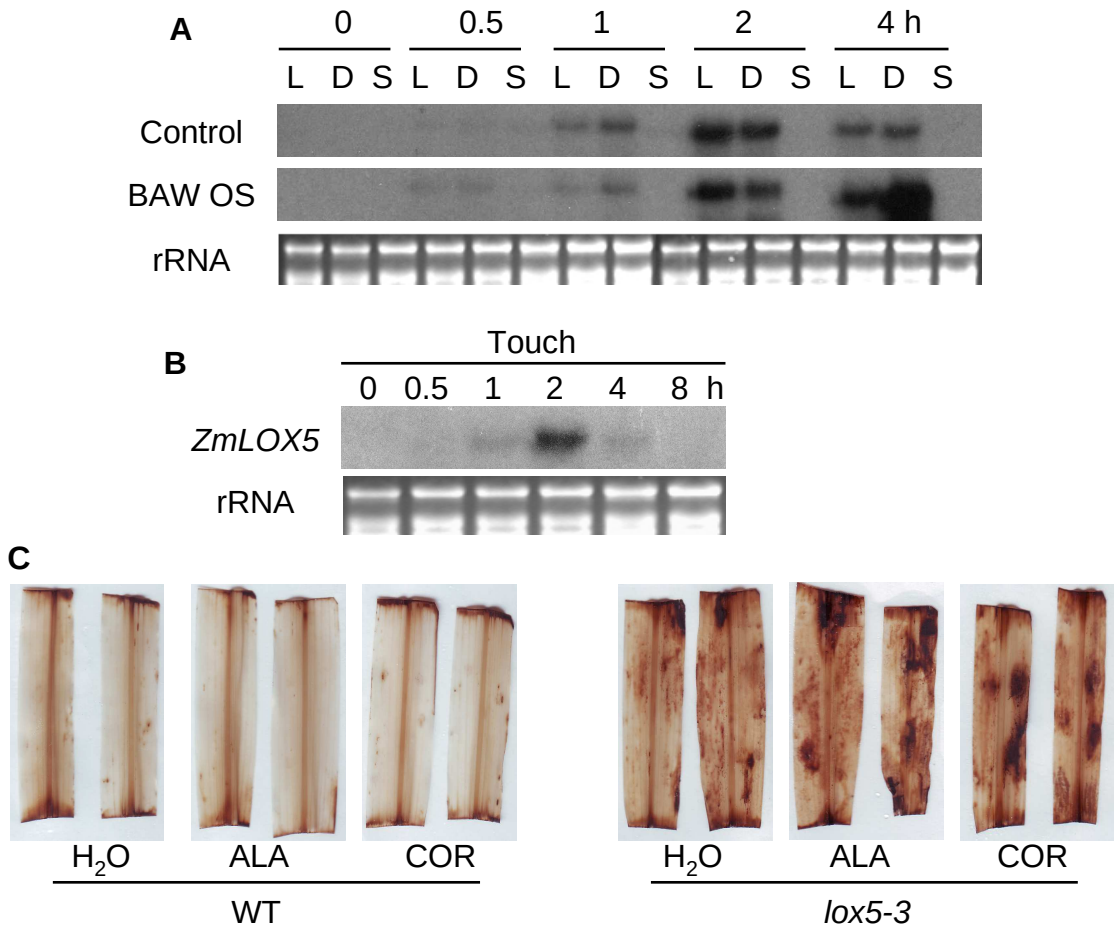


**Figure 17**. Expression of *ZmLOX5* in response to beet armyworm oral secretion and touch stimulus, and quantification of hydrogen peroxide levels in response to diverse treatments.

A, Leaves of B73 seedlings were wounded and with BAW oral secretion or wáter was applied to the wound side. RNA was extracted from local leaves (L, wounded site), distal portion of the wounded leaf (D, upward for the wound site) or systemic leaves (S, upper leaf). B, RNA was extracted from the leaves that were gently bent and stroked, at designated time points. Fifteen µg of total RNA was loaded into each lane of 1.5% formaldehyde RNA gel and separated by electrophoresis, transferred onto nylon membranes, and hybridized with $P^{32}$-labeled gene-specific probes. Ethidium bromide staining of gels confirmed equal loading of RNA samples. C, Hydrogen peroxide was detected in WT and *lox5-3* mutant treated with water, alamethicin (ALA) and coronitine (COR) by using a DAB staining

## DISCUSSION

Our data provided strong genetic evidence for the important role that a 9-LOX-mediated metabolism has in plant defense to insect herbivory. Thus far, such a role has been widely ascribed to 13-LOX final metabolites, jasmonates and GLVs, in Arabidopsis, tobacco, tomato, and other dicot species (Bell et al., 1995; Heitz et al., 1997; Haltischke and Baldwin 2003; Zheng et al., 2007). However, in addition to these two groups of lipid-derived signals, there are multitude of other plant oxylipins that are synthesized either constitutively or in a stress-inducible manner by the initial action of 9-LOXs on linolenic or linoleic acid substrates (Fig. 8). Our understanding of the physiological functions of these so called 9-oxylipins is still rudimentary.

9-LOXs have been primarily implicated in plant defenses to pathogen infection (Weber et al., 1999; Göbel et al., 2001, 2002; Ponce de Leon et al., 2003; Hamberg et al., 2003). In agreement with such a role, our recent results of the functional analyses of maize knock-out 9-LOX mutant *lox3-4*, this mutant is substantially more susceptible to root-knot nematodes and the seed-infecting fungi *Aspergillus* spp. (Gao et al., 2008a, 2009). In contrast, this same mutant is more resistant to several other maize fungal pathogens including stalk rotting *Fusarium verticillioides* and *Colletotrichum graminicola* as well as leaf blighting *Cochliobolus heterostrophus* (Gao et al., 2007; Isakeit et al., 2007). These data indicate that, depending on specific pathogens, 9-LOXs may have either a defense role or even facilitate pathogenesis and disease progression.

Prior to this study, few expression or oxylipin profiling studies indicated potential role of 9-oxylipins in plant response to insect feeding. For example, 9-HPODE content increased in potato after aphid infestation (Gosset et al., 2009).

Because of the existence of multigene subfamilies of 9-LOXs (Park et al., 2010) that collectively produce a large number of reported 9-oxylipins, in this study, we have attempted to identify 9-oxylipin species specifically produced by the *ZmLOX5* isoform. For this, we compared oxylipin profiles of *lox5-3* mutant and NIL WT in response to mechanical damage. Among the oxylipins measured, accumulation of 9-HPOTE and 9-HOTE was induced in wounded WT leaves (Fig. 13A) suggesting that they are candidate 9-oxylipins mediating defense against insect attack. Surprisingly, although difference was observed between WT and the mutant, only moderate reduction of these metabolites in the *lox5* mutant after wounding was detected (Fig. 13A). To test whether other functional 9-LOXs may have compensated for the lack of *ZmLOX5*, transcripts of four other maize 9-LOXs were measured in wounded WT and *lox5-3* mutant. Northern blot analyses showed that *ZmLOX1*, *ZmLOX2* and *ZmLOX4* were slightly overexpressed in the *lox5* mutant when compared with WT (Fig. 14). Therefore, we hypothesize that these three genes may be responsible for wound-induced levels of 9-products in the *lox5* mutant.

Since the relatively small reduction in 9-oxylipins detected in the *lox5* mutant is unlikely to be responsible for the dramatic increase in insect susceptibility, we have quantified 13-oxylipins including the major defense hormone JA. Ample evidence demonstrates that jasmonates play a pivotal role in plant defense responses against insect

herbivory (Howe and Jander, 2008). Remarkably, the *lox5* mutant was unable to produce wild type levels of wound-induced JA (Fig. 15). This is an intriguing finding since recombinant ZmLOX5 exhibited 94% 9-LOX activity in vitro (Park et al., 2010). Reduced production of wound-induced JA was accompanied by attenuated wound-inducible expression of one of the previously characterized 13-LOX gene, *ZmLOX10* (Nemchenko et al., 2006). Taken together, these results indicate that *ZmLOX5* is required for normal induction of JA in response to mechanical damage. We speculate that reduced wound-induced levels of JA may be responsible for the observed decreased resistance of the *lox5* mutants to beet armyworm infestation. These data also demonstrate remarkable interconnectivity between the signaling pathways mediated by diverse 9- and 13-LOXs, which appear both synergistic (Z*mLOX5* vs *ZmLOX10*) and antagonistic (*ZmLOX5* vs other 9-LOXs).

Another unexpected finding of this study was that of moderately increased ET emission in the *lox5* mutant in response to wounding, which was corroborated by increased wound-induced expression of the ET biosynthesis gene, *ACO31,* in wounded *lox5* mutant leaves as compared to WT (Fig. 16). ET has been reported to play a key role in plant defense mechanisms in response to biotic- and abiotic stresses (Johnson and Ecker, 1998; Lin et al., 2009). The function of ET in plant defense to insect herbivory is still largely controversial (van Loon et al., 2006). For example, ET has been implicated in increased susceptibility to aphid in alfalfa (Dillwith et al., 1991), barley (Miller et al., 1994) and wheat (Anderson and Peters, 1994). In contrast to these reports, ET is shown to be associated with increased resistance in barley-aphid interaction (Argandoña et al.,

2001). Although this study does not provide conclusive evidence to support either positive or negative roles of ET in insect defense, it appears to be associated with increase susceptibility to insect feeding.

In the course of our phenotypic analyses of the *lox5* mutants, we observed consistently elevated levels of $H_2O_2$ in mechanically stimulated *lox5* mutant leaves (Fig. 17C). This prompted us to test whether *ZmLOX5* expression is mechano-sensitive as has been shown for other touch-inducible genes from diverse plant species (Mizoguchi et al., 1996; Oh et al., 1996; Mauch et al., 1997; Tatsuki and Mori, 1999). Indeed, *ZmLOX5* was induced by mild wounding both locally and distally and touching stimulus (Fig. 17A and 17B). Because of increased production of $H_2O_2$ in response to touch stimulus observed in *lox5* mutant leaves, it is tempting to speculate that one potential function of touch-inducible expression of *ZmLOX5* is to prevent abnormally high levels of accumulation of $H_2O_2$ in response to mild damage of tissue. Interestingly, van Breusegem et al., (2001) showed that $H_2O_2$ induces biosynthesis of ET in plant tissues, suggesting a potential link between increased ET and $H_2O_2$ observed in our study.

In summary, this study provides strong genetic evidence for the role the 9-LOX, *ZmLOX5*, in maize insect defense and implicates, for the first time, 9-LOX metabolism in the regulation of JA biosynthesis in response to mechanical damage.

## MATERIALS AND METHODS

### Identification of *Mu*-insertional mutant alleles of *ZmLOX5*

Mutator transposon insertional mutants were identified by PCR bases screening of the maize reverse genetics resource [Trait Utility System for Corn (TUSC)] at Pioneer Hi-Bred Intl as described by Gao et al., (2007). For this, we used *Mu* specific primer 9242 based on the conserved terminal inverted repeat sequences of the Mutator transposable elements and *ZmLOX5*-specific primers (Table 3). Three independent alleles (*lox5-1*, *lox5-2* and *lox5-3*) were identified and PCR fragments flanking the insertion sites were cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced to identify the precise location of the *Mu*-insertions. As a rule, original mutants contain a number of unrelated *Mu*-elements elsewhere in the genome. To remove these unwanted mutations, the original mutant alleles were backcrossed to the B73 inbred line at least five times to seven times and self-pollinated to generate the $F_2$ populations that segregated for the mutants alleles. These individuals were then screened by genotyping PCR and Southern blot analyses at the $BC_{5\text{-}7}F_2$ genetic stage to identify near-isogenic (NIL) wild type or mutant lines. Although, mutant alleles were created in Tx714, CML176, W438, Tx772 and FR2128 genetic backgrounds, in this study the NILs in B73 genetic background were used.

**Oral secretion of beet armyworm (BAW, *Spodoptera exigua*) application on leaves**

Oral secretion extraction from beet armyworm (BAW) was performed as described previously (Engelberth et al., 2007). The third leaves of the V3 developmental stage in the B73 genetic background were scratched (referred to as mild wounding) with a razor blade and 3 μl of extracted oral secretion was quickly applied to the wounded area. For control treatment, water was applied to wounded area. For the expression analyses shown in Figure 17, leaf tissues were taken from oral secretion treated area (approximately 5-6 cm, local), distal area (upward leaf from local area), and fourth leaves (upper leaves, systemic). All samples were harvested, immediately frozen in liquid $N_2$, and stored at -80 $^{\circ}$C for further RNA extraction and northern blot analyses.

**Mechanical wounding and touch stimulation of leaves**

For wounding experiments, second leaves of the V2 stage seedlings at $BC_7F_3$ stage in B73 background were wounded by crushing the leaf blade with a hemostat perpendicular to the main vein (avoiding damage to the main vein) and the entire wounded leaf was harvested at specific time points as described by Park et al., (2010). For all experiments, each replicate consisted of at least two seedlings and three replicates were analyzed for each time point.

For touch stimulation of leaves, the third leaf of the V3 stage of W438 seedlings were gently bent and stroked back and forth with a hand 10-15 times as followed by

previous research (Tatsuki and Mori, 1999). Treated leaves were incubated and harvested at designated time points (0, 0.5, 1, 2, 4 and 8 h), and immediately frozen in liquid $N_2$, and stored at -80 $^{\circ}$C. W438 was used in this experiment because this inbred line responds to insect feeding with greater levels of both GLV and terpenoid volatile synthesis than B73 (Jim Tumlinson, upublished data). We reasoned that since volatile organic compounds are primarily produced in plant trichomes, touch stimulating of this line would result in the greater response of touch-sensitive genes.

**Quantification of 9- and 13-oxylipins, jamonic acid (JA) and ethylene (ET)**

Oxylipin profiling and ET emission monitoring was performed on the leaves of WT and *lox5-3* mutant in B73 background at the $BC_7F_3$ genetic stage. Leaves (0.6-1.0 g) of V3 seedlings were wounded by crushing the leaf blade with hemostat perpendicular to the main vein (avoiding any damage cause to the veins) at room temperature and harvested at 0, 4 and 8 h after wounding. Selected 9- and 13-oxylipins were measured as described in Park et al., (2010). Five biological replicates were analyzed per genotype with each replicate containing leaves from two seedlings.

For JA analysis, second leaves of V2 stage seedlings of were wounded as described above, and harvested at the time points (0, 1, 4, and 8 h). Wounded plant seedlings were then frozen in liquid $N_2$ immediately. Five biological replicates were used for JA quantification. JA levels were determined by using a liquid chromatography/mass spectrometry (LC/MS) method as described by Pan et al., (2008)

with following modifications. After wounding, approximately 100 mg of leaf tissues were placed in a 2 ml screw-cap Fast-Prep tube (Qbiogene, Carlsbad, CA, USA) with the standard dihydro JA (10-50 ng) and 0.5-1 g of Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, USA). Prepared samples were then homogenized and shaken for 30 min at 4 ºC. Homogenized samples were added with dichloromethane (1 ml), shaken for 30 min at 4 ºC, and centrifuged for 5 min at 13,000 g. The bottom organic phase was then transferred into 1.8 ml glass vials (VWR International, West Chester, PA, USA), evaporated by continuous air flow, and then dissolved in 300 µl of methanol. JA analyses were performed by using a QTrap 2000 (Applied Biosystems, Foster, CA, USA) LC/MS with a Discovery C18 HPLC column (5 cm×2.1, 5µm particle size; Supelco, Bellefonte, PA, USA) and a flow rate of 100 µl $min^{-1}$.

Ethylene was measured from fourth leaves of 3-week-old seedling (V4 stage) by mechanical wounding as described above, and quantification of ethylene was carried out by using the method described by Gao et al., (2008) with some modifications. Excised leaf tissues were collected and weighted at the time points (0, 1, 2, 4, 8 and 12 h) after wounding. Leaf segments (approximately 10 cm) were incubated in a glass vials individually to accumulate ethylene for three hours. One ml of the headspace gas was withdrawn from the vials by a syringe and analyzed using gas chromatography. Five replicates were used per each genotype.

**Isolation of genomic DNA and Southern blot analysis**

For Southern blot analysis, V2 stage seedlings of individual of the WT, heterozygote and mutant in B73 line were used for extraction of genomic DNA as described by Zhang et al., (2005), and genomic DNA (10 µg) of each NIL was then digested by *Eco*RV overnight at 37 °C. Separation of digested DNA was carried in a 0.8% agarose of a TAE-based electrophoresis gel. Separated genomic DNA was transferred with 25 mM phosphate transfer buffer (pH 6.5) to a nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN, USA) overnight and then cross-linked to the nylon membrane by a UV cross-linker. The blots were hybridized overnight at 42 °C with the $^{32}$P-labelled *ZmLOX5* gene specific probe (primer sequences were listed in Table 3) in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA), and washed according to the manufacturer's instructions.

**RNA extraction and northern blot**

Total RNA was extracted using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Ten to 15 µg of RNA was loaded in 1.5% (w/v) formaldehyde agarose gel in 1X MOPS buffer for separation of RNA and was transferred onto a nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN, USA). To verify equal loading of RNA in gel, all RNA samples were confirmed with an ethidium bromide under UV light for all

experiments. Membranes were hybridized with 50 to 100 ng of $^{32}$P-labelled gene-specific probes in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) at 45 °C for overnight. Washes were performed twice with 2X SSC, 0.1% SDS for 15 min at 65 °C and twice with 0.1X SSC, 0.1% SDS for 20 min at 65 °C. Membranes were then dried at room temperature for 30 to 40 min, and were exposed to a BioMax X-ray film (Kodak, Rochester, NY, USA) in a cassette. All experiments were independently conducted at least twice.

**Semi-quantitative reverse transcription polymerase chain reaction**

Wounded leaves from WT and mutant seedlings were harvested at designated times. Total RNA samples were treated with RNase-free rDNase at 37 $^{o}$C for 30 min by using a DNA-free kit (Ambion Inc., Austin, TX). First strand cDNA (5 µg of RNA as a template per each sample) was synthesized by following a First-Strand Synthesis Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) protocol. The cDNA was denatured at 94 $^{o}$C for 5 min and amplified by following 27-32 cycles (each cycle: 45 sec at 94 $^{o}$C, 1 min at 56 $^{o}$C, and 2 min at 72 $^{o}$C). Amplified PCR products were loaded and separated on 1.2-1.5% agarose gels. All primers for RT-PCR in this study are listed in Table 3. As an internal control, expression of a constitutively expressed house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH,* GeneBank accession number: X07156) was used.

**Beet armyworm infestation**

In excised leaves, B73 background (WT and mutant) seedlings at $BC_7F_3$ stage were grown to the V3 stage. Third leaves were cut and placed in a tray and were infested by third-instar larvae of BAW. Five replicates of plant per host genotype, two plants per replicate, were used for this assay. Larvae weight was measured two days post infestation.

In intact leaves, V3 developmental stage of the NILs in B73 background plants at the $BC_7F_3$ stage were infested by second to third instar stage of larvae of BAW contained in a cage on leaves for three days at room temperature. Weight gain was calculated based on the weight difference between larvae before and after infestation. Seven replicates were examined per host genotype. Infested area with BAW larvae was scanned and analyzed by using the ImageJ sofeware (ImageJ 1.36b; Wayne Rasband, NIH, Bethesda, MD, USA).

**Detection of $H_2O_2$ by DAB staining**

Leaf segments were excised, water, alamethicin (ALA, 10 µg/ml) and coronitine (COR, 0.5 µg/ml) dropped, and placed in DAB solution (3,3-diaminobenzidine-HCl, pH 3.8, 1mg/ml) with low vacuum pressure for 30 min, and leaves were incubated for overnight at room temperature as described by Park et al., (2005). DAB polymerizes to produce a brown precipitate on contact with $H_2O_2$. Subsequently, the leaves were cleared

in alcoholic lacto-phenol (2:1:1, 95% ethanol:lactic acid: phenol) at 65 $^{o}$C for 1 h. The samples were then washed with 50% ethanol and were scanned.

# CHAPTER IV

# SUBFUNCTIONALIZATION OF THE TWO SEGMENTALLY DUPLICATED 9-LIPOXYGENASES *ZMLOX4* AND *ZMLOX5* OF MAIZE IN RESPONSE TO PATHOGEN INFECTIONS

## INTRODUCTION

*Colletotrichum graminicola* [teleomorph *Glomerella graminicola*] belongs to the *Ascomycetes* and is the causal agent of maize anthracnose leaf blight (ALB) and stalk rot disease (Bailey and Jeger, 1992). In addition to being recognized as one of the most damaging maize pathogens, *C. graminicola* has been a model system to study plant-pathogen interactions because it is a haploid organism and is relatively easily manipulated by using genetic approaches, including gene disruption and transformation (Epstein et al., 1998; Thon et al., 2000). In addition, this fungus is one of the best studied maize hemibiotrophs that first grows as a classical biotroph within the host tissues and then switches its lifestyle to necrotrophy as it continues to infect the host (Herbert et al., 2004).

Another economically important maize fungal pathogen is *Aspergillus flavus* (Samson, 1992). The major economic losses are caused by *A. flavus* ability to infect oil-rich seed and produce the highly carcinogenic mycotoxins aflatoxins (Scheidegger and Payne, 2003). *A. flavus* mainly produces aflatoxin $B_1$ and $B_2$ and cyclopiazonic acid (CPA), and some strains of *A. flavus* can additionally produce aflatoxin $G_1$ and $G_2$

(Cardwell and Cotty, 2002). Besides *A. flavus*, another seed colonizing pathogen is *Fusarium verticillioides.* This fungus produces another highly toxic class of toxins called fumonisins (Shim and Woloshuk, 2001).

It has been noted that, at least for *A. flavus*- infected seed, most of the crops that accumulate high levels of these mycotoxins are oil-rich crops, including maize, peanut, cotton, and tree nuts, indicating that plant lipids may have a crucial role in the plant-fungal interactions (Diener et al., 1987; Keller et al., 1994). There are at least two hypotheses that have been proposed to explain the connection between lipids and aflatoxin biosynthesis. The first possibility comes from the findings that acyl-CoA precursors of aflatoxin may be provided through mitochondrial and peroxisomal β-oxidation of plant-derived fatty acids in the fungus (Maggio-Hall et al., 2005). The second hypothesis postulates that host-derived, oxidized lipids function as signal molecules that can stimulate aflatoxin production by the fungus (Brodhagen and Keller, 2006; Christensen and Kolomiets, 2011). For example, the level of aflatoxin production was increased when the synthetic medium, which *A. flavus* and *A. parasiticus* was grown, was supplemented wiht linoleic acid (Fabbri et al, 1983; Passi et al., 1984). Also, plant-derived methyl-jasmonic acid can stimulate aflatoxin production and suppress aflatoxin production by *A. parasiticus* either in static YES medium or in A&M medium, respectively (Burow et al., 1997; Vergopoulou et al., 2001). In addition, green leaf volatiles produced by the LOX pathway have been implicated in the inhibition of aflatoxin production by *A. flavus* on seeds of maize, cotton and peanut (Zeringue, 1991).

These results suggest that the products of the LOX pathway may govern the interactions between host seed and mycotoxigenic fungi.

In support of the idea that lipids are important factors in seed susceptibility to mycotoxin accumulation (Christensen and Kolomiets, 2011), previous research has shown that disruption of oxylipin synthesis mediated by LOXs in maize results in altered susceptibility of the host to either infect the host and/or accumulate mycotoxins (Gao et al., 2007, 2009). Specifically, mutation of a maize 9-LOXs, *ZmLOX3*, resulted in a reduction of the number of conidia and levels of fumonisin $B_1$ produced by *F. verticillioides* on infected kernels. The mutant was also more resistant to the leaf fungal pathogens *C. graminicola* and *Cochliobolus heterostrophus* (Gao et al., 2007). However, the same mutant was more susceptible to *Aspergillus* spp. (Gao et al., 2009). These findings suggest that LOX-derived oxylipins governs the outcome of plant fungal interaction and this effect is pathogen-species specific. Herein, I report the functional relevance of two other maize 9-LOXs, *ZmLOX4* and *ZmLOX5,* and their metabolites in host interactions with *C. graminicola, A. flavus* and *F. verticillioides*.

## RESULTS AND DISCUSSION

### The *lox4* mutant alleles are loss-of-function alleles of *ZmLOX4*

I explained the generation of the near-isogenic wild-type (WT) and mutant of the *ZmLOX4* were similar to those used for *ZmLOX5* (CHAPTER III). Four independent

mutant alleles were identified for the *ZmLOX4* gene (Fig. 18A). The original *lox4* mutant alleles were back crossed into several genetic backgrounds including B73. *Mutator* insertions were confirmed by Southern blot analysis. The homozygote mutant and WT displayed different band sizes, suggesting the presence of *Mu* insertion in the gene (Fig. 18B). Because the *ZmLOX4* was highly expressed in shoot apical meristem (SAM), expression of this gene was measured in SAM in *lox4-7* and *lox4-10* mutants. As expected, the transcripts of *ZmLOX4* were strongly expressed in near-isogenic WT plants, whereas no discernable transcript was detected for exonic insertion events in the *lox4-7* and *lox4-10* mutants (Fig. 18C). These data demonstrate that *lox4-7* and *lox4-10* mutants are strong null alleles of *ZmLOX4*. In all functional analyses, the near-isogenic mutant and WT lines at the $BC_{5-7}$ genetic stage were examined. To identify whether there was any interaction between the pathways initiated by *ZmLOX4* and *ZmLOX5* isoforms, *lox4-10 lox5-3* double mutants were generated in the B73 genetic background.

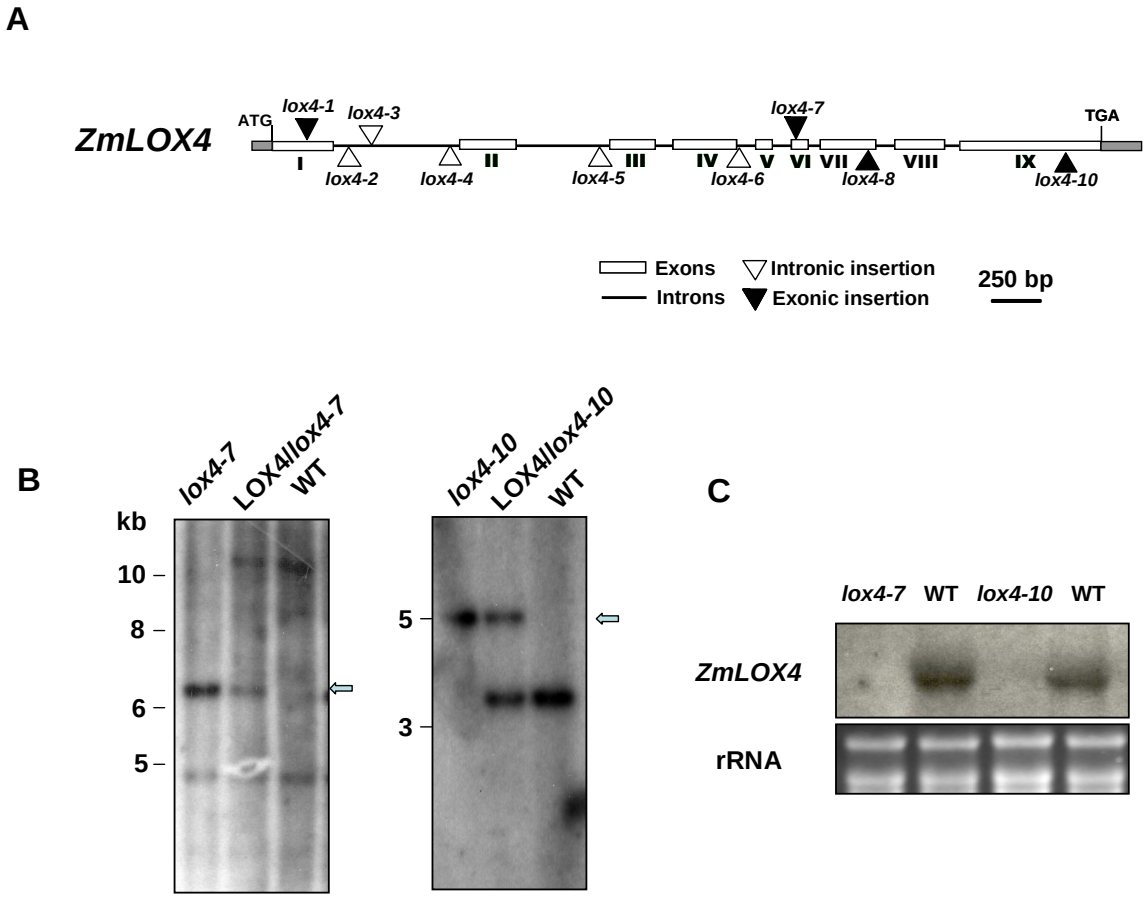


**Figure 18.** The *lox4* alleles are strong loss-of-function alleles of the *ZmLOX4* gene.

A, Schematic representation of the genomic structure of the *ZmLOX4* indicating *Mutator* element insertional sites (shown as triangles). B, Southern blot analysis showing restriction size polymorphism in due to insertion of *Mu*-elements. Genomic DNA (15 µg per lane) digested with with *Eco*RV and *Kpn*I (*lox4-7* and *lox4-10*, respectively) loaded on the gel and hybridized with *ZmLOX4* gene specific probe. The arrows represent the mutant band. WT, wild-type near-isogenic sibling; *lox4-7* and *lox4-10*, homozygous mutant; LOX4/*lox4-7* and LOX4/*lox4-10*; heterozygous individuals. C, Northern blot analysis of the *ZmLOX4* transcripts in shoot apical meristems of *lox4* mutant alleles and corresponding near-isogenic wild type.

**The *lox4* mutants are more susceptible but *lox5* mutants are more resistant to *C. graminicola***

To determine whether *ZmLOX4* or *ZmLOX5* have a function in defense against *C. graminicola*, leaves of single and double mutant seedlings were inoculated with *C. graminicola* conidia as described in Gao et al., (2007). As shown in Figure 19A, *lox4-10* was more susceptible to *C. graminicola* when compared to the WT as evidenced by increased lesion area (Fig. 19A and 19B). In contrast to the *lox4* mutants, *lox5* mutant alleles displayed increased resistance to this pathogen and this phenotype was confirmed for the *lox5* alleles in B73 genetic background. Interestingly, *lox4-10 lox5-3* double mutant displayed disease symptoms that appeared to be intermediate between the single mutants, suggesting that *ZmLOX4* and *ZmLOX5* may antagonistically interact with each other.

Since *C. graminicola* is a major stalk rotting pathogen in maize, the effect of mutantions of these genes in disease progression in stalk tissues was examined. Similar to anthracnose leaf blight phenotypes, *lox4-7* and *lox4-10* mutant alleles displayed visibly greater rotted areas compared to the near-isogenic WT plants (Fig. 20A). As shown by scoring the lesion area distribution, approximately 60% of the *lox4* mutants showed large lesion areas (>150 $mm^2$), whereas only 10% of lesions formed in the WT stalks were larger than 150 $mm^2$ (Fig. 20B). In contrast to the enhanced susceptibility of the *lox4* mutants, disruption of the *ZmLOX5* gene resulted in significantly decrease in stalk rot lesion area (Fig. 21A) with no large lesions formed on the *lox5* mutants, while

approximately 40% of the lesions formed in NIL WT stalks were larger than 600 $mm^2$ (Fig. 21B). Together with the results of the tests for susceptibility to anthracnose leaf blight, these findings suggest that these two closely-related duplicated paralogs have evolved opposite functions in relation to maize interactions with *C. graminicola*. More specifically, *ZmLOX4* appears to have a clear defense-related role, whereas *ZmLOX5* mediated metabolism facilitates the process of pathogenesis. Additional comparative molecular and biochemical analyses of the two mutants and WT will be required to shed light to such a differential function of these two duplicated genes in response to infection with *C. graminicola*.

One of the potential mechanisms underlying the opposing phenotypes observed for the two mutants may be related to the differential activation of salicylic acid (SA) pathway. The importance of SA signaling in plant defense to *Colletotrichum* spp. has been reported previously (O'Donnell et al., 2001; Lee et al., 2009). The present study, although the levels of SA were not measured, I found that pathogenesis-related proteins (PR-1 and PR-5) were overexpressed in the *lox5* mutants infected with *C. graminicola*. It is possible that *lox5* mutant produces higher levels of SA in response to infection and *lox4* mutant may be characterized by lower levels of SA. To test this hypothesis, additional experiments will be needed to measure SA in all host genotypes after inoculation with this fungus.

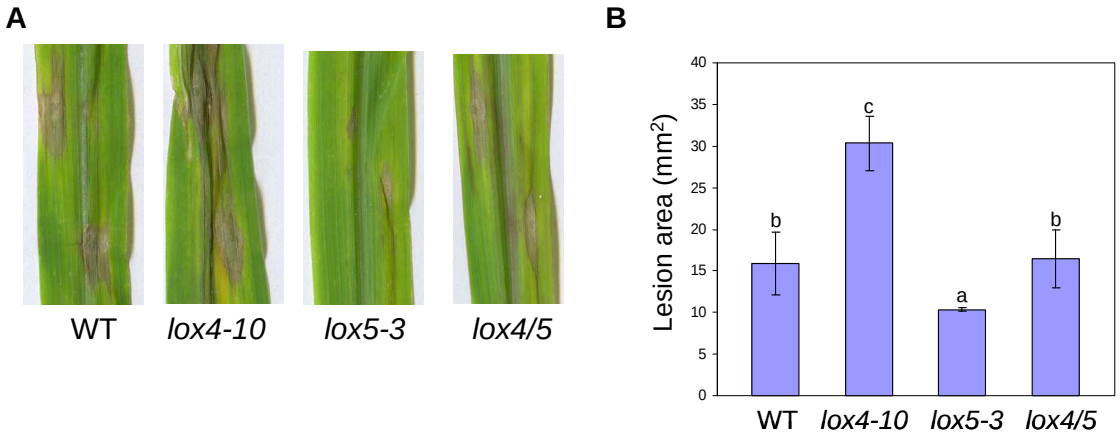


**Figure 19.** The *lox4* and *lox5* mutant phenotyes to anthracnose leaf blight caused by *Colletotrichum graminicola*. The third leaves of V3 stage in B73 genetic background were inoculated with 10 µl of spore suspension of *C. graminicola* (M1.001).

A, Disease symptom on each genotype in response to inoculation with *C. graminicola.* The leaves were excised and scanned to produce digital images at five days post inoculation. B, Lesion area after scanning of leaf tissues was measured by using the ImageJ software. The data represented as means ± SD (n=6). All experiments were repeated at least three times and similar results were presented at independent experiment.

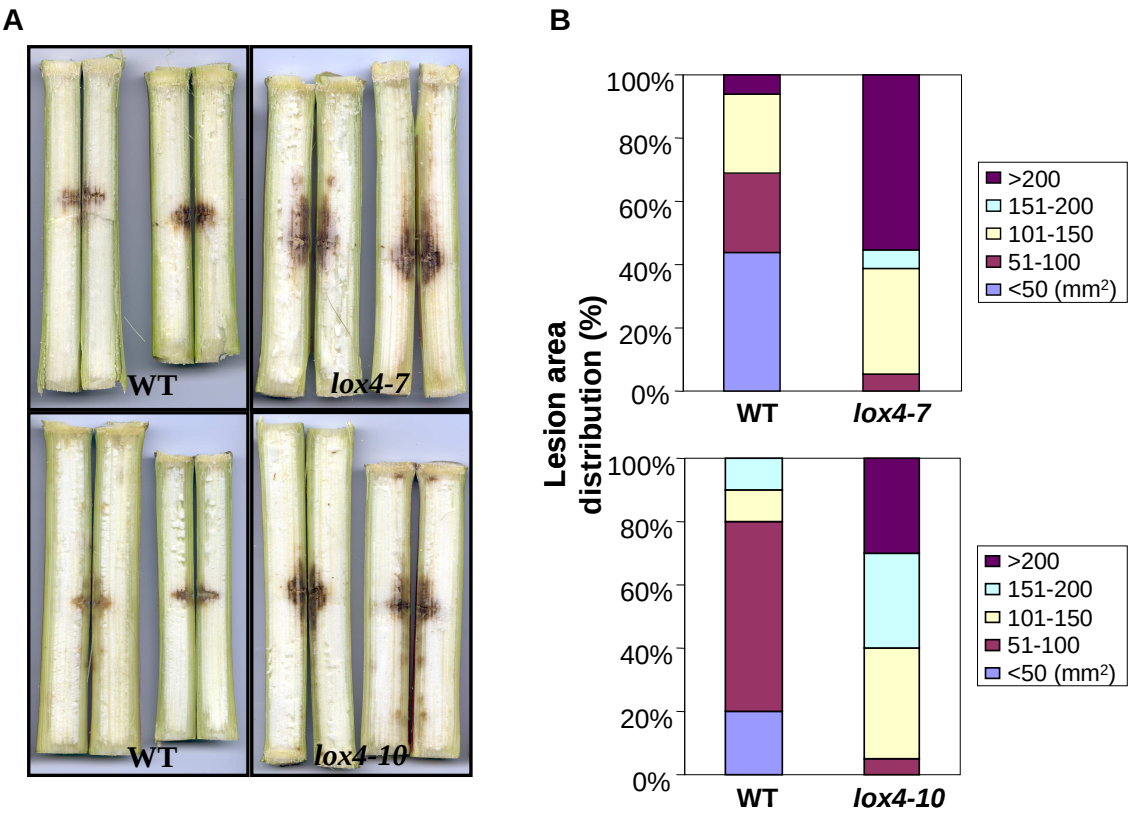


**Figure 20.** Stalk rot assays on wild-type (WT) and two *lox4* mutants in response to *Colletotrichum graminicola*.

A, Stalks were scanned 10 days post inoculation in WT and *lox4-7* and *lox4-10* mutants. B, Quantified lesion areas by using the ImaeJ software were categorized five different classes: <50, 50-100, 101-150, 151-200 and >200 $mm^2$.

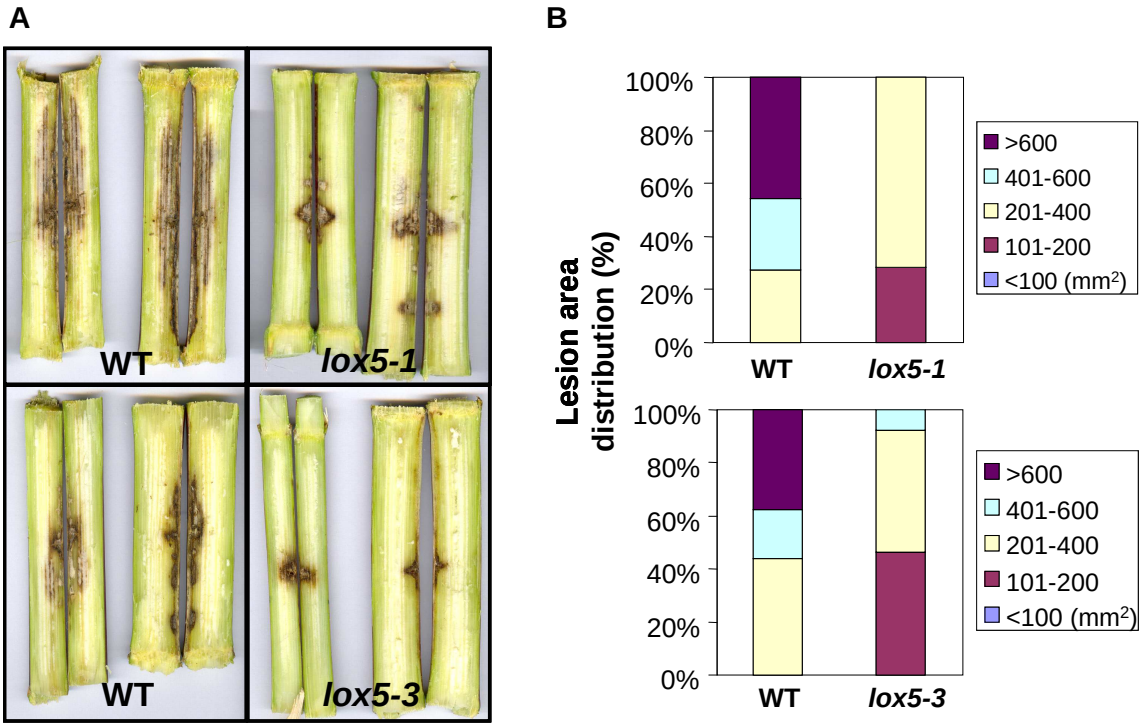


**Figure 21.** Stalk rot assays on wild-type (WT) and two *lox5* alleles in response to *Colletotrichum graminicola*.

A, Stalks were scanned 10 days post inoculation in WT and *lox5-1* and *lox5-3* mutants. B, Quantified lesion areas by using the ImaeJ software were categorized five different classes: <100, 101-200, 201-400, 401-600 and >600 $mm^2$.

**Alteration of some 13-LOX derived oxylipins but not 9-LOX products in response to *C. graminicola***

To identify oxylipins produced specifically by the ZmLOX4 and ZmLOX5 isoforms in response to infection by *C. graminicola*, amounts of several major 9- and 13-oxylipins in the infected *lox4* and *lox5* mutants and WT leaves were measured. The following oxylipins were measured by HPLC 9-hydro(pero)xy-octadecadienoic acid (9-H(P)ODE), 9-hydro(pero)xy-octadecatrienoic acid (9-H(P)OTE), 13-hydro(pero)xy-octadecadienoic acid (13-H(P)ODE), and 13-hydro(pero)xy-octadecatrienoic acid (13-H(P)OTE) were measured. Surprisingly, the levels of 9-oxylipins were not statistically different between infected WT and either *lox4* mutant or *lox5* mutants at the time points tested (Fig. 22A).

Because both isoforms were 9-LOXs (Park et al., 2010), it was surprising that the *lox4* mutant produced moderately but significantly lower levels of 13-HPOTE and 13-HOTE at 2 days post inoculation when compared to the WT ($P<0.05$). In contrast, 13-HPOTE accumulated to higher levels in the *lox5* mutant at 4 days as compared to the WT (Fig. 22B). These data indicate that the two mutants are affected in their ability to regulate synthesis of certain 13-oxylipins either negatively (*lox4* mutant) or positively (*lox5* mutant). 13-HPOTE and 13-HOTE showed anti-fungal activity in oilseed rape previously (Graner et al., 2003).

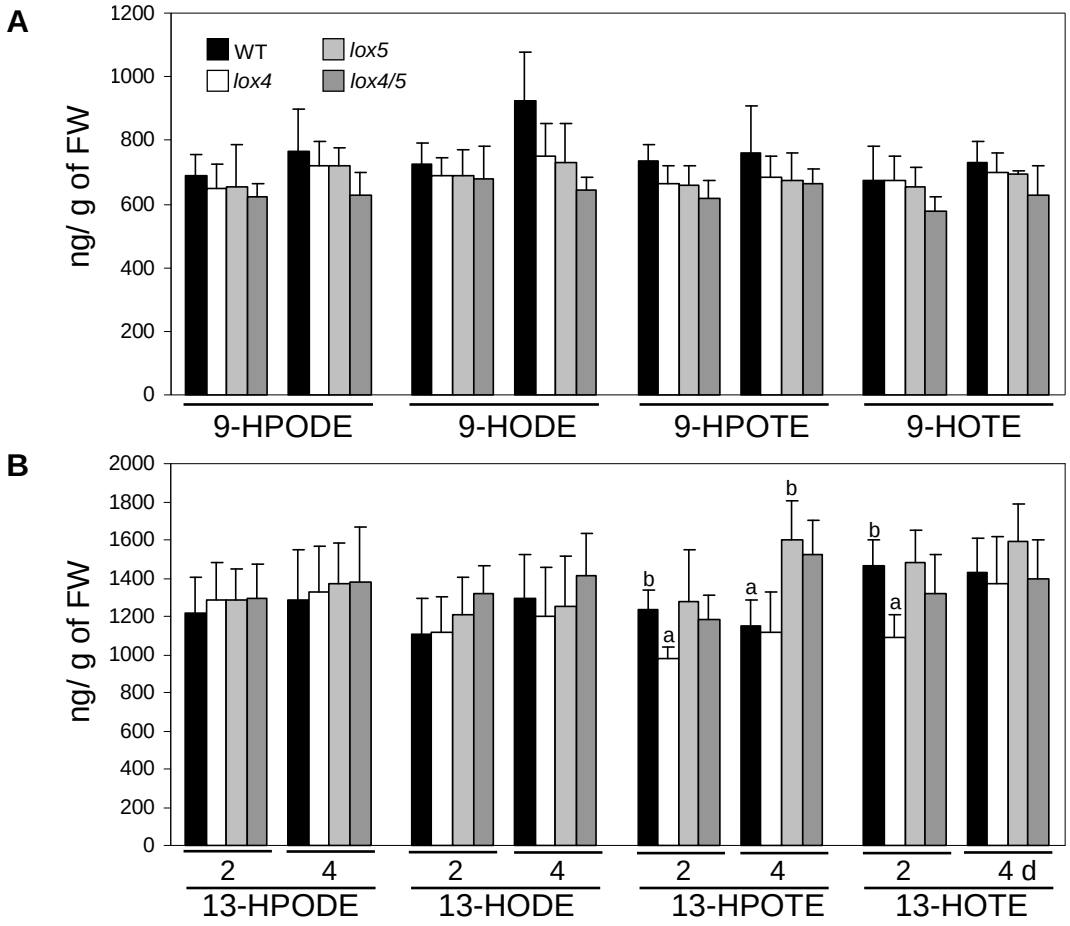


**Figure 22**. Content of 18:2 and 18:3 derived 9- and 13-oxylipins extracted from wild-type (WT, black bar), *lox4* mutant (white bar), *lox5* mutant (gray bar) and double mutant (dark gray bar) at 2 and 4 days post inoculation with *Colletotrichum graminicola*.

A, 9-HPODE, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid; 9-HODE, (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid; 9-HPOTE, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecadienoic acid; 9-HOTE, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroxy-10,12,15-octadecatrienoic acid. B, 13-HPODE, (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid; 13-HODE, (9*Z*,11*E*,13*S*)-13-hydroxy-9,11-octadecadienoic acid; 13-HPOTE, (9*Z*,11*E*,13*S*,15*Z*)-9-hydroperoxy-9,11,15-octadecatrienoic acid; 13-HOTE, (9*Z*,11*E*,13*S*,15*Z*)-9-hydroxy-9,11,15-octadecatrienoic acid. Letters above bars denote significant differences ($P<0.05$) using a SPSS program.

Therefore, it is possible that increased resistance of the *lox5* mutants to *C. graminicola* is due to increased production of 13-HPOTE in the *lox5* mutant at 4 days post inoculation (Fig. 22B), and that lower than WT induction of 13-oxylipins in the *lox4* mutants may be the reason behind its enhanced susceptibility to *C. graminicola.*

**_ZmLOX4_ and _ZmLOX5_ have different roles in response of kernels to _A. flavus_**

Since one of the major goals of our laboratory is to elucidate the role of oxylipins and LOXs in maize resistance to contamination of seed with mycotoxins, I have tested *lox4* and *lox5* mutants for their interaction with aflatoxin-producing *A. flavus* by using both a laboratory-based kernel assay as well as field-based tests. As Figure 23A illustrates, the *lox5* mutant kernels were heavily colonized by *A. flavus* compared to WT. Consistent with the increase in fungal colonization, infected *lox5* mutant kernels produced significantly more conidia at 7 days post inoculation (Fig. 23B). In contrast to the *lox5-3* mutant, the fungus grew poorly on the *lox4-10* mutant (Fig. 23A) and a three fold reduction of conidia number was observed in the *lox4-10* mutants (Fig. 23B). Although the levels of colonization and conidiation of the *lox4* and *lox5* mutants were dramatically altered, no statistically significant difference in the accumulation of aflatoxin was detected in both mutants compared with WT (AF, Fig. 23C).

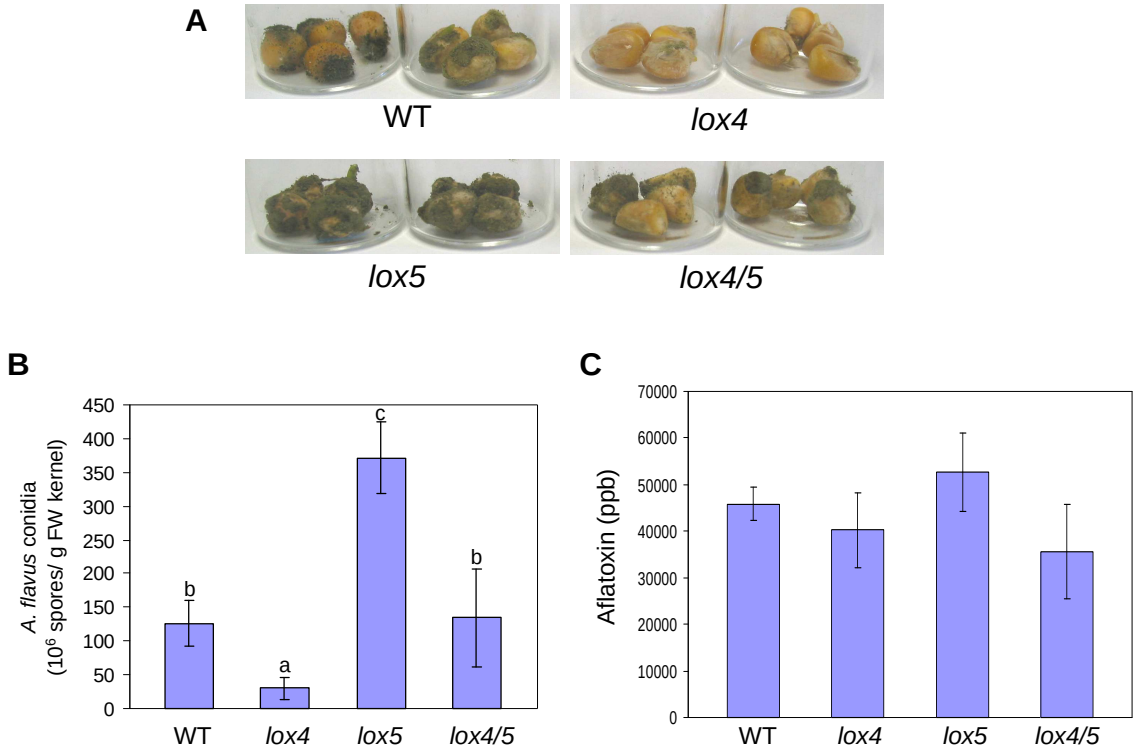


**Figure 23.** Kernel bioassays in wild-type (WT), *lox4-10*, *lox5-3* single mutant and *lox4-10 lox5-3* double mutant to *Aspergillus flavus* NRRL 3357.

Sterilized kernels from each genotype were inoculated with 200 µl of spore suspension of *A. flavus* ($10^6$ conidia/ ml) and incubated for seven days. Visual differences in fungal colonization (A), production of asexual spores (B) and aflatoxin (AF) production were performed. Similar results were obtained at least three times. Different letters above bars denote significantly different between genotypes ($P<0.05$, SPSS).

Remarkably, double mutants showed WT levels of fungal growth and conidiation thus canceling whichever effects single mutations had on these two pathogenicity processes of fungal infection. Once again, these results clearly indicate that these duplicate genes have opposing functions during host interaction with this pathogen.

**AF accumulation was reduced in *lox5* mutants but not in *lox4* mutants in the field**

Although no changes of AF were observed in the kernel assays for the two mutants, AF accumulation was measured under the field conditions in the College Station 2006 to 2008 growing seasons. In these tests, the *lox5-2* mutant was backcrossed at least 4 times into the genetic backgrounds of three inbred lines which were well characterized in the corn breeding program at Texas A&M University, Tx714, FR2128 and the check aflatoxin resistant line Tx772. Remarkably, in all three genetic backgrounds of the *lox5* mutants displayed two to five times reduction of AF levels and similar reduction of AF was reproducibly observed for other *lox5* mutant alleles in B73 background in following years (Fig. 24). In contrast, the levels of AF in *lox4* mutants were similar to the WT levels (data not shown).

It may seem inconsistent that under the field conditions the *lox5* mutants accumulated significantly lower levels of AF, because in the kernel assays, no difference in the AF accumulation levels was observed between *lox5* mutant and WT kernels.

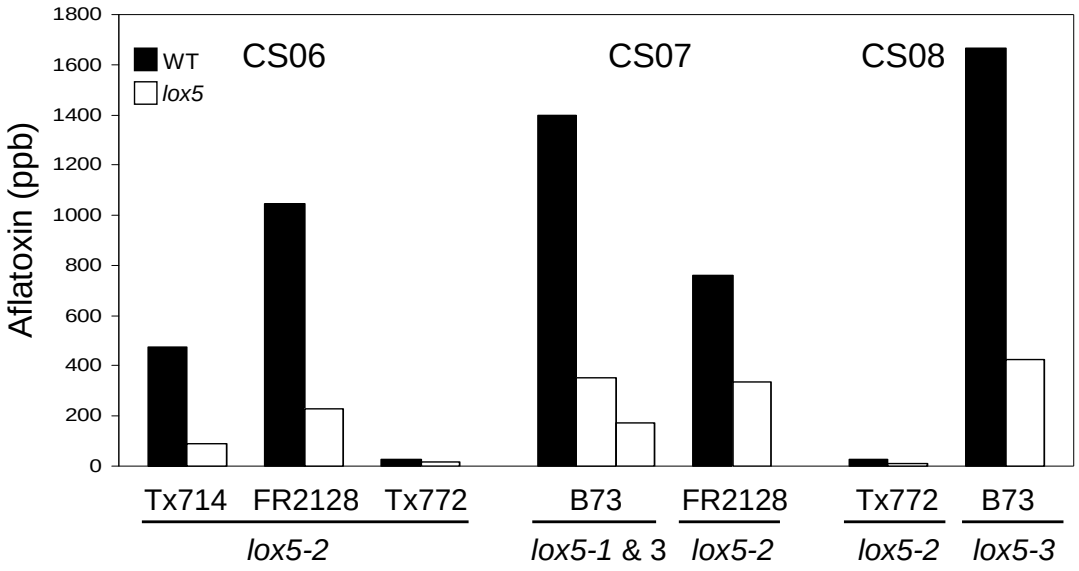


**Figure 24.** The *lox5* mutants accumulated significantly lower levels of aflatoxin in the field. Several different genetic backgrounds of wild-type (WT, black) and *lox5* mutants (white) were arraged in a randomized complete black design containing four lows (~15 plants each row) and inoculated 10 days after mid-silking with 3 ml of $3x10^7$ of *Aspegillus flavus* NRRL 3357. The aflatoxin was quantified with a VICAM aflatoxin fluorometer USDA-FIGS protocol.

One probable explanation for such a difference is that under the field conditions the fungal spores were inoculated via silk channel (a natural route for fungal infection of maize cobs and kernels) and *A. flavus* grew down the silks to the kernels. Thus, I speculate that silk is an important barrier for fungal infections of cobs and its defense mechanisms may be important to prevent accumulation of AF in maize kernels. To date, the molecular and biochemical mechanisms of the *ZmLOX5* mutation in the reduction of AF contamination in maize are unknown. However, one potential mechanism is that silks of the *lox5* mutant may produce some anti-mycotoxin compounds after inoculation of this fungus (i.e. antioxidant compounds).

**Colonization, sporulation and fumonisin $B_1$ production by *F. verticillioides* on the *lox4* and *lox5* mutants**

To examine the role of the *ZmLOX4* and *ZmLOX5* genes in seed interaction with *F. verticillioides*, I performed maize kernel infection bioassays to quantify fungal colonization, conidiation and $FB_1$ production. The *lox4* and *lox5* mutants displayed substantially increased susceptibility to colonization by *F. verticillioides*, which was accompanied by up to a three to five fold increase in the number of microconidia on the two mutants as compared to WT (Fig. 25A and 25B).

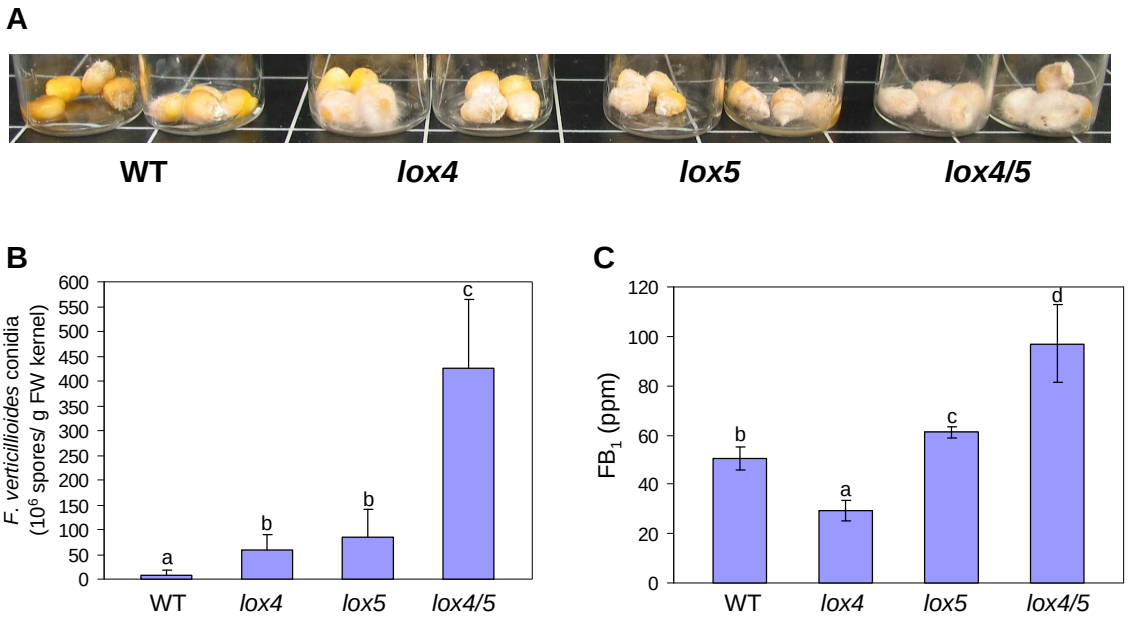


**Figure 25.** Colonization, conidiation and fumonisin $B_1$ production by *Fusarium verticillioides* on the wild-type (WT), *lox4*, *lox5* and double mutants.

A, Comparison of visual colonization by *F. verticillioides*. The pictures were taken at seven days post inoculation. B, Number of conidia produced on infected kernels of each genotype seven days after inoculation. C, Fumonisin $B_1$ ($FB_1$) was quantified seven days after inoculation using high-performance liquid chromatography (HPLC). The values are the mean±SD of four replicates per host genotype. Different letters above bars denote significantly different means ($P < 0.05$) analyzed by the SPSS program between genotypes.

Unlike colonization and conidiation, *lox4* mutant kernels supported lower levels of $FB_1$ production, although higher levels of $FB_1$ accumulated in the *lox5* mutant (Fig. 25C). Notably, the *lox4-10 lox5-3* double mutant was more susceptible to fungal colonization as evidenced by dramatically greater production of both conidia and $FB_1$, suggesting that two genes have additive effect on kernel resistance to colonization by *F. verticillioides*.

**Ethylene (ET) partially induces fungal growth in the two gene kernels infected by *A. flavus* and *F. verticillioides***

ET has been broadly reported to play important roles in regulating diverse physiological and defense processes of plants including seed germination, organ senescence, abscission and fruit ripening (Kende, 1993; Johnson and Ecker, 1998), and responses to abiotic- and biotic stresses, such as wounding, chilling, drought, flooding, hypoxia, ozone and pathogen attack (Lin et al., 2009). In addition, a recent study showed that both Arabidopsis mutants with reduced ET signaling or perception and a RNAi silenced ET mutant of wheat are more resistant to *F. graminearum* than WT (Chen et al., 2009), suggesting that *F. graminearum* may exploit ET signaling to aid colonization of both dicot and monocot hosts.

Therefore, I hypothesized that *F. verticillioides* and *A. flavus* might manipulate ET production in seeds. I reasoned that one potential mechanism underpinning altered resistance levels to the two fungi may be altered production of ET. To test this hypothesis, ET was measured in seeds infected by *A. flavus* and *F. verticillioides.* As described in the methods, each genotype was inoculated with the fungal spores or mock-inoculated and incubated until 3 and 6 days post inoculation. As Figure 26A demonstrates, *lox4* mutant produced lower levels of ET compared with the WT at 3 days but higher levels of ET in *lox5* mutant was detected at 3 days post inoculation with *A. flavus*. In addition, both *lox4* and *lox5* mutants resulted in increased ET production after inoculation with *F. verticillioides* at 3 days (Fig. 26B). The heavily grown fungi induced higher ET emission in two single mutants but it was not positively correlated in the double mutant. These data suggest that fungal pathogens may manipulate the ET synthesis in kernels to facilitate their growth and pathogenicity processes such as conidia production.

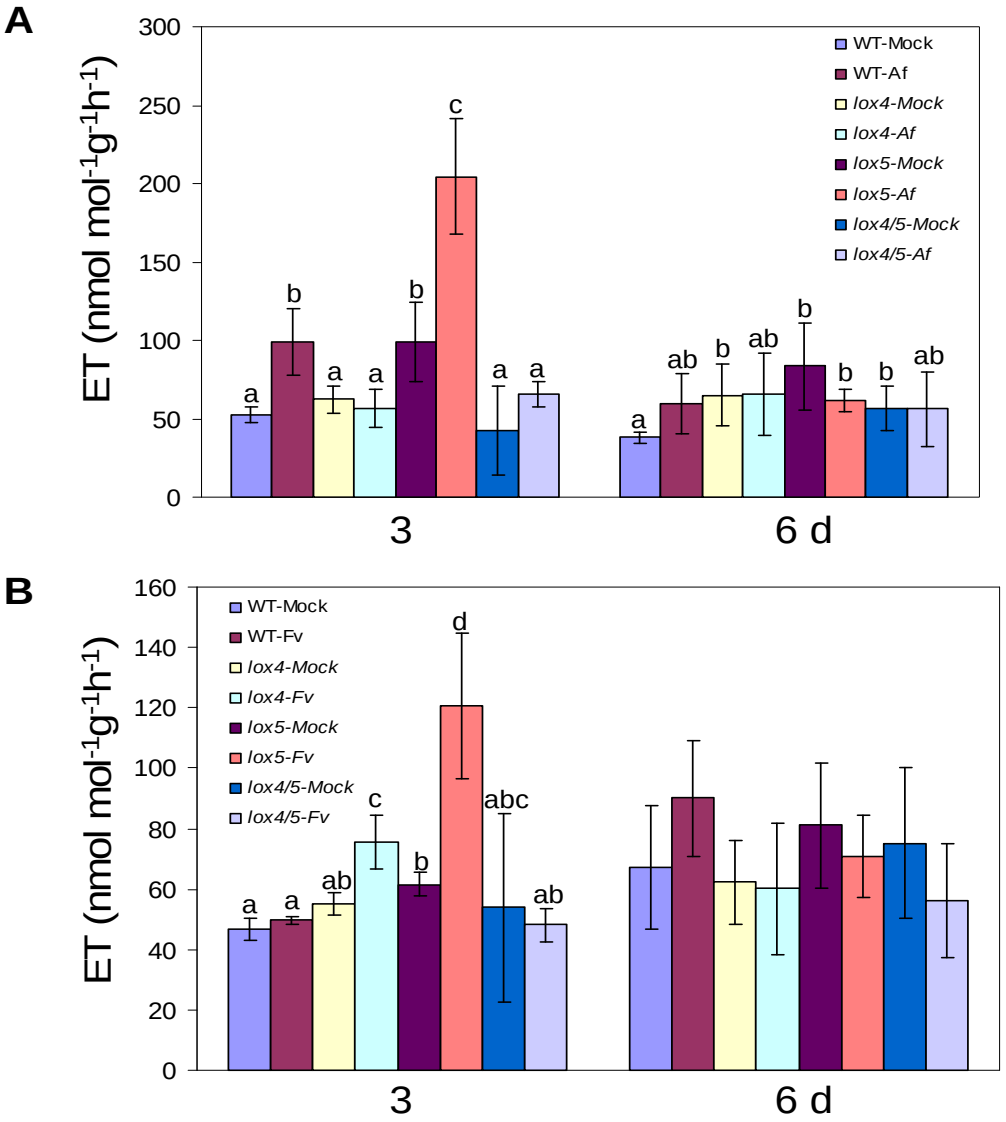


**Figure 26.** Ethylene (ET) production by the wild-type (WT), *lox4*, *lox5* and double mutants infected with *Aspegillus flavus* and *Fusarium verticilliodes*.

A, ET was measured at 3 and 6 days post-inoculation with either control or 200 µl of *A. flavus* suspension ($10^6$ spores/ ml). B, ET was quantified at 3 and 6 days time points in response to either control or 200 µl *F. verticilliodes* ($10^6$ spores/ ml). The vials were tightly sealed for 3 hours prior to withdrawal of headspace gases to allow detection of ET accumulation. The values are the mean±SD of five replicates per genotype. Different letters above bars denote significantly different means ($P < 0.05$, ANOVA) analyzed by the SPSS program between the host genotypes within same time point.

## MATERIALS AND METHODS

### Plant materials

The generation of near-isogenic wild-type (WT) and mutant alleles by using a reverse genetics resource [Trait Utility System for Corn (TUSC)] used at Pioneer Hi-Bred Intl as described in CHAPTER III. The *ZmLOX4* near-isogenic WTs and mutants were screened by using *Mu* (*Mutator*) specific prime 9242 (5'-AGAGAAGCCAACGCCAWCGCCTCYA-3') in combination with either of the *ZmLOX4*-specific primers: 4-1F 5'-CTTTGCTCGCCGCCACATCACATT-3', 4-1R 5'-GGGAGTAGAGATTGTGCGGGTAGAT-3', 4-7F 5'-GTTCCTCAGAAGCATTCTGAACGAT-3', 4-7R 5'-CAAGTTGCCAGACGTGGCCCTCAG-3', 4-8R 5'-CCAGCTCTTGTACACGTCGGAG-3', 4-10F 5'-GCCGGACCAGTCAAGCCCCTAC-3' and 4-10R 5'-ATCTACAAACCATCCGCTCAGGC-3'

### Isolation of genomic DNA and southern blot analysis

For Southern blot analysis, V2 stage seedlings of individual of the wild-type (WT), heterozygote and mutant in the B73 line were used for extraction of genomic DNA as described in CHAPTER III. Genomic DNA (10 µg) was then digested by

*Eco*RV (*lox4-7*) and *Kpn*I (*lox4-10*) for overnight at 37 °C. Separation of digested DNA was in a 0.8% agarose of a TAE-based electrophoresis gel. Separated genomic DNA was transferred with 25 mM phosphate transfer buffer (pH 6.5) to a nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN, USA) for overnight and then cross-linked to the nylon membrane by a UV cross-linker. The blots were hybridized overnight at 42 °C with the $^{32}$P-labelled *ZmLOX4* gene specific probe (primer sequences were listed in CHAPTER II) in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA), and washed according to the manufacturer's instructions.

**RNA extraction and northern blot**

Harvested shoot apical meristem tissues were immediately frozen in liquid $N_2$, and stored at -80°C. Total RNA was extracted using TRI reagent (Molecular Research Center Inc., Cincinaati, OH, USA) according to the manufacturer's protocol. Fifteen μg of RNA was loaded in 1.5% (w/v) formaldehyde agarose gel in 1X MOPS buffer for separation of RNA and was transferred onto a nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN, USA). To verify equal loading of RNA in gel, all RNA samples were confirmed with an ethidium bromide under UV light for all experiments. Membranes were hybridized with 50 ng of $^{32}$P-labelled *ZmLOX4* gene-specific probe in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) at 45 °C for overnight. Washes were performed as described in CHAPTER III.

**Anthracnose leaf blight and stalk rot assays with inoculation of *C. graminicola***

For the anthracnose leaf blight assay, methods were modified from as described in Gao et al., (2007). The V3 to V4 stage seedlings in B73 genetic background of wild-type (WT), *lox4* mutant, *lox5* mutant and *lox4/5* double mutant were used for this assay. Plant were laid down on the wet paper towels in trays and inoculated with six to eight 10 µl of suspension of *C. graminicola* M1.001 ($10^6$ spores/ml). Inoculated plants were incubated in a humidity tray covered with Press-N-Seal (The Glad Products Company, Oakland, CA, USA) until 24 h at room temperature. Twenty-four hours after inoculation, the seal was removed from a humidity tray and the seedlings were allowed to dry before being returned to upright position. The inoculated plants were placed under a 16 hr photoperiod (120 µmol $m^{-2}$ $s^{-1}$; Quantum Mete, Apogee Instruments, Logan, UT, USA) at 25 to 28 °C. The leaves were excised and scanned for disease development and scanned, and the lesion areas were quantified at five days after inoculation by using the ImageJ software (ImageJ 1.36b; Wayne Rasband, NIH, Bethesda, MD, USA).

The stalk rot assays were carried out following the methods of Gao and associates (2007) with some modifications. The stalks of 10-week-old plants under greenhouse condition at 22 to 28 °C were wounded with a needle (1 cm in depth) and immediately inoculated with approximately 150 µl suspension of *C. graminicola* M1.001 ($10^6$ spores/ml) on the wound sites with a cotton swab. After inoculation, plants were then sealed with a parafilm to develop disease on stalks for 10 days. Harvested stalks were spilt longitudinally and scanned for measurement of lesion areas.

**Fungal inoculation and spore counting on kernels by *A. flavus* and *F. verticillioides***

For inoculation with *A. flavus* NRRL 3357 and *F. verticillioides* 7600 (M3125), methods followed those described by Gao et al., (2007) with minor modifications. Seeds were surface-sterilized with bleach (containing 6% sodium hypochlorite) for 10-15 min and rinsed with sterilized, distilled $H_2O$ at least five times. The embryos of kernels were cut longitudinally using a razor blade to a depth of about 0.5 cm to provide an infection court for fungal inoculation. Seeds were then blotted dry with paper towel and placed in a 20 ml glass scintillation vial (Wheaton Science, Millville, NJ) and inoculated with 200 µl of $1\times10^6$ conidia of *A. flavus* NRRL 3357 or 200 µl of $1\times10^6$ conidia of *F. verticillioides* 7600, containing 0.001% Tween 20 per vial. Control seeds (mock) received an equal amount of 0.001% Tween 20. Four inoculated or mock-treated kernels were used per replicate with at least four replicates per experiment. The inoculated kernels were kept in a plastic transparent container with wet filter paper to provide humidity and incubated with 12 h light/day at 26-29 °C for seven days post inoculation. Sterile, distilled $H_2O$ was added to containers as needed to maintain high humidity.

To measure levels of conidia production, infected kernels were placed in a 20 mL glass vial with 2 mL of 0.001% Tween 20, and vortexed for 20 seconds to dislodge spores. The spore suspension was decanted and spores were enumerated using a hemacytometer.

**Quantification of aflatoxin, fumonisin $B_1$**

Infected or mock-treated kernels from each treatment were ground using a Waring blender (Waring laboratory, Torrington, CT) and aflatoxin was subsequently quantified with a fluorometer using the VICAM AflaTest® USDA-FGIS procedure (VICAM, Watertown, MA). Kernels infected by *A. flavus* were ground and extracted with 20 ml of 80% (vol/vol in water) methanol using the blender. Fumonisin $B_1$ measurement was conducted using an HPLC as described previously (Gao et al., 2007). Mycotoxin concentrations in ng/g were log-transformed to equalize variance. Data were subjected to an analysis of variance and Fisher's protected LSD test was used to separate means.

**AF contamination levels under field conditions**

In addition to AF contamination under lab conditions, quantification of AF in fields was conducted for three years at the Texas Agricultural Experiment Station in College Station, Texas. Each replicate consisted of 10-15 plants and four replicates were tested for each genotype arranged in a randomized complete block design. At ten-days post-mid silking, primary ears were inoculated with 3 ml of suspension of $3\times10^7$ conidia of *A. flavus* by using a non-wounded silk channel method (Zummo and Scott 1992). Infected ears were harvested when kernel moisture dropped below 15%. The extraction of AF followed above.

**Oxylipin profiling**

The B73 genetic background seedling at V4 stage was inoculated with *C. graminicola* as described above. Leaves were harvested at designated time points (0, 2 and 4 days) and immediately placed in liquid $N_2$. The methods were as described in CHAPTER III.

**Measurement of ethylene produced in infected kernels by *A. flavus* and *F. verticillioides***

Ethylene (ET) produced by maize kernels infected with *A. flavus amd F. verticillioides* per genotype was quantified as described by Gao et al., (2008) with some modifications. Briefly, the vials containing infected kernels were kept at 12 h light/day at 26-29 °C and ET was measured at 3 and 6 days post-inoculation (dpi). Vials were sealed with screw caps with septa. One ml of the headspace gas was withdrawn from vials by a syringe and analyzed using gas chromatography.

# CHAPTER V

# GENERAL CONCLUSION

The primary objective of my Ph.D research project was to elucidate the biochemical and physiological roles of two 9-lipoxygenase genes, *ZmLOX4* and *ZmLOX5* in maize to diverse stresses. The genes were differentially expressed in various maize organs and tissues as well as in response to diverse stress treatments. The transcripts of *ZmLOX4* accumulated predominantly in roots and shoot apical meristem, whereas *ZmLOX5* was expressed in most tested aboveground organs. Both genes were not expressed in untreated leaves, but displayed differential induction by defense-related hormones. While *ZmLOX4* was only induced by jasmonic acid (JA), the transcripts of *ZmLOX5* were increased in response to JA and salicylic acid (SA) treatments. *ZmLOX5* was transiently induced both locally and systemically by wounding and fall armyworm herbivory, suggesting a putative role for this gene in defense against insects. Surprisingly, despite of moderate JA- and wound-inducibility of *ZmLOX4*, the gene was not responsive to insect herbivory. These results suggest that the two genes may have distinct roles in maize adaptation to diverse biotic- and abiotic stresses.

To test the physiological role of Zm*LOX5* in maize defense against insect herbivory, I generated several *Mutator*-insertional knock-out alleles of this gene. Disruption of this gene resulted in reduced levels of several 9- as well as 13-LOX derived oxylipins in response to wounding. Most notably, *lox5* mutants displayed significant reduction in wound-induced JA. Corroborating the idea that *ZmLOX5*-derived

9-oxylipins regulate wound-induced biosynthesis of JA, expression of several putative JA-producing 13-LOX genes was suppressed in the *lox5* mutant in response to wounding. Beet armyworm feeding assays using detached and intact leaves showed increased insect performance and reduced resistance of *lox5* mutant compared to near-isogenic wild type. Decreased insect resistance of the mutants has been consistently observed under field conditions. Therefore, this study provides strong genetic evidence for the importance of the 9-oxylipins in maize defense to insect herbivory via regulating the JA biosynthesis.

In contrast to the mutant interactions with insects, functional analyses of the *lox4* and *lox5* mutants uncovered that, despite their extreme sequence similarity, the two paralogs have evolved opposite functions in maize interactions with pathogens. While *lox4* mutants were dramatically more susceptible to anthracnose leaf blight and stalk rot caused by *C. graminicola*, *lox5* mutants displayed decreased anthracnose disease symptoms. The double mutants displayed wild type resistance levels indicating that biosynthetic and/or signal transduction pathways mediated by these two genes are antagonistic to each other. Supporting divergent roles for the two genes in plant-pathogen interactions, the kernels of *lox4* and *lox5* mutants showed opposing phenotypes related to *A. flavus* colonization of see. While *lox5* supported greater seed colonization and production of spores by *A. flavus*, *lox4* seed was more resistant to this pathogen. However, the two mutants showed similar phenotypes in colonization and conidiation in response to *F. verticillioides*. Multiple alterations in the oxylipin signature were detected in WT, each single and the *lox4 lox5* double mutants in response to leaf infection with *C.*

*graminicola.* While 13-oxylipins were upregulated in *lox5* mutant, *lox4* mutant accumulated low levels of 13-oxylipins. Interestingly, the alteration of 13-oxylipins in leaves infected by *C. graminicola* was differential compared to that caused by mechanical wounding. Altogether, our data suggest that the two genes have evolved opposing functions in resistance to pathogens versus insects and that *ZmLOX4* may play a key role in plant-pathogen interactions but *ZmLOX5* may be involved in host defenses to insect herbovory.

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