UNRAVELING RECIPROCAL LIPID- MEDIATED COMMUNICATION BETWEEN MAIZE SEED AND *ASPERGILLUS FLAVUS*

A Dissertation

by

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ABSTRACT

It is generally accepted, that the reciprocal exchange of molecules between plants and fungi govern the outcome of their interaction. From a multitude of potential signals, one class of oxidized lipids (oxylipins) has taken central stage in this concept. Synthesized from enzymatic and non-enzymatic peroxidation of fatty acids, oxylipins are a large and diverse group of potent endogenous signaling molecules. Because plant and fungal oxylipins are similar biochemically and structurally, a novel hypothesis has emerged that during plant-fungal interactions, that these metabolites are exchanged, perceived, and affect the behavior of opposing partner. This study sought to explore this hypothesis and assign a role to specific oxylipin-producing enzymes from maize and *Aspergillus flavus* within the context of the oxylipin-mediated cross-kingdom crosstalk.

Maize wild-type and near-isogenic mutants for several lipoxygenase (LOX) and 12-oxophytodienoate reductases (OPR) related to jasmonic acid biosynthesis and nine oxylipin-mutant strains of *Aspergillus flavus* were utilized to investigate the contribution of oxylipins from the plant and the fungi on fungal ability to colonize the host, sporulate and produce aflatoxin. Phytohormone content and gene expression analyses of infected seed were preformed to explore potential associations of defense hormones with fungal pathogenesis processes.

Results showed that several genes involved in JA biosynthesis affect specific fungal processes. Despite belonging to separate subgroups, both LOX3 and LOX7 are

involved in defense against colonization. Additionally, *LOX3* expression correlates negatively with colonization and *lox3* knockout mutants are more susceptible regardless of the fungal genotype indicating a general defensive role for LOX3 against *A. flavus* colonization. An unexpected major finding from this study is that JA biosynthesis genes appear to promote aflatoxin accumulation.

Results provide evidence that the fungal LOX gene is required for normal colonization of seed, while PpoA is required for normal conidia and aflatoxin production. The ratio of ABA/JA correlates positively with aflatoxin accumulation.

These findings are expected to expedite studies of seed-fungal interactions, lead to uncovering novel regulators of seed defense, find specific host genes and signals that regulate conidiation and mycotoxin production, and eventually provide the maize industry with genetic targets and biochemical markers for selecting aflatoxin resistant lines.

DEDICATION

To my father, Eliborio Borrego Jr., and

mother, Maria Magdalena Borrego

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

The intimate interactions between plants and other organisms are regulated by a multitude of small organic molecules originating from both interacting organisms. Recently oxygenated fatty acids, termed oxylipins (Gerwick et al., 1991), have taken center-stage as potent endogenous and exogenous signaling molecules and have been proposed to regulate the complex interactions between plants and fungi (Tsitsigiannis and Keller, 2007; Gao and Kolomiets, 2009; Christensen and Kolomiets, 2011; Borrego and Kolomiets, 2012). In this hypothesis, the biochemical and structural similarities between plant- and fungal- derived oxylipins allows reciprocal exchange, perception, and specific response to these molecular signals, the processes that eventually govern whether the fungus can effectively colonize the plant and whether the fungus be able to reproduce and synthesize secondary metabolites, including health hazardous, mycotoxins. This dissertation explores oxylipin-mediated communication in the agro-economically important plant-fungal interaction of maize seed with the mycotoxigenic pathogen, *Aspergillus flavus*.

Biosynthesis of oxylipins proceeds through both enzymatic and non-enzymatic processes; however, fatty acid oxygenases are the primary sources of oxylipins in both plants and fungi (Fig. 1). Initiation of biosynthesis begins with the liberation of fatty acids from cellular membranes through lipase activity. Recent studies showed that fatty

acids esterified to complex membrane lipids might also be used as substrates for further oxidation by diverse fatty acid oxygenases (Andreou et al., 2009; Brodhun and Feussner, 2011) . Temporal-spatial specific mono- and di-oxygenases can then incorporate molecular oxygen into the hydrocarbon tail to produce hydroxy- and hydroperoxy- fatty acids, which can then be incorporated into subsequent downstream branches of oxylipin metabolism.

The lipoxygenase (LOX) pathway is the major producer of oxylipins in plants (Feussner and Wasternack, 2002) and utilizes both linoleic (18:2) and linolenic (18:3) acids for substrate. LOX isoforms are grouped broadly into two classes (9-LOX and 13-LOX) dependent on the regio-specificity of oxygenation carbon position. The maize inbred line B73 (Schnable et al., 2009) contains thirteen LOX isoforms (ZmLOX1 – ZmLOX13). These isoforms are classed as 9-LOX (ZmLOX1, 2, 3, 4, 5, 12) (Gao et al., 2008b; Park et al., 2010) and 13-LOXs (ZmLOX7, 8, 9, 10, 11) (Nemchenko et al., 2006; Acosta et al., 2009; Christensen et al., 2013) members. Further, the maize genome also contains an unusual hydroperoxide lyase-like LOX isoform, ZmLOX6 (Gao et al., 2008a). The peroxidation of linoleic acid yields hydroperoxy-octadecadienoic acid (HPOD), while peroxidation of linoleic acid produces hydroperoxy-octadecatrienoic acid (HPOT). These hydroperoxides can then be used as substrates for at least six distinct branches of oxylipin metabolism (allene oxide synthase (AOS), divinyl synthase, epoxy alcohol synthase, hydroperoxide lyase (HPL), lipoxygenase (LOX), and peroxygenase) or reduced to their hydroxy derivatives (9/13- HOT/D).

The best studied branches of plant oxylipin metabolism are the 13-LOX-derived AOS and HPL which produce jasmonates (Wasternack, 2007; Wasternack and Hause, 2013) and green leaf volatiles (GLVs) (Matsui, 2006; Scala et al., 2013), respectively. Jasmonates are a subgroup of oxylipins comprised of the developmental and defensive phytohormone jasmonic acid (JA), its derivatives, and precursors. The first member and the group's namesake was first identified over half a century ago from the floral scent volatiles of the jasmine flower (*Jasminum grandiflorum* L.) (Demole et al., 1962) and since then, this oxylipin group has become the best characterized in plants.

To date, the only oxylipin receptors identified in plant species belong to the jasmonate subgroup. The first plant oxylipin ligand discovered was the JA-isoleucine conjugate and its receptor, the JA ZIM domain repressor proteins (JAZ)-SCF^{COI1} complex (Chico et al., 2008). In the absence of JA-Ile, JAZ proteins act as transcriptional repressor by virtue of binding to JA-responsive transcription factors (e.g., MYC2). Following JA-Ile binding, SCF^{COI1}, an E3-ubiquitin ligase, targets JAZ proteins for degradation through the 26S proteasome mechanism. This derepresses the transcription factors allowing for subsequent transcription of JA-responsive genes. More recently putative receptor for 12-oxo-phytodienoic acid (12-oxo-PDA) was identified (Park et al., 2013) as a chloroplast-localized cyclophilin that is involved in retrograde signaling. The cyclophilin-OPDA complex binds serine acetyl-transferase and increases the production of cysteine and glutathione that relay the signal to transcription factors. Receptors for other oxylipins remain to be identified, and inquiry into diverse receptor classes is ongoing, e.g., animal oxylipins are perceived through G-protein coupled

receptors (GPCRs) (Bos et al., 2004). Additionally, many oxylipins are reactive electrophilic species and may not require receptor-ligand binding for activity (Farmer and Davoine, 2007; Mueller and Berger, 2009).

Similar to plants, fungal-derived oxylipins are produced primarily through oxygenase activity, but much less is known about their biosynthesis, perception, or activity (Andreou et al., 2009; Brodhun and Feussner, 2011). A fungal oxylipin producing enzyme with similar function, but phylogenetically unrelated to LOX, is the linoleate diol synthase (LDS), also known as Ppo (Psi producing oxygenase), which catalyzes the formation of Psi (precocious sexual inducer) factors. These were initially identified for their regulation of sexual and asexual sporulation in fungi (Champe and el-Zayat, 1989). The *Aspergillus flavus* genome contains four Ppo paralogs (Brown et al., 2009) and one lone LOX gene (Horowitz Brown et al., 2008) (Fig. 1).

This study aimed to examine the role of selected members of the maize LOX gene family during the oxylipin-mediated cross-kingdom communication with *A. flavus* (Fig. 2). The LOX and OPR genes were selected because they were shown or suspected to directly produce JA (*LOX7*; this study, OPR7 and OPR8; Yan et al. 2012) or indirectly by producing yet to be identified regulators of JA biosynthesis (LOX3; Gao et al. 2009; LOX5; not published and LOX10; Christensen et al. 2013). Despite rapid advancements in chemical and genetic crop technologies, aflatoxin produced by *A. flavus* continues to threaten the maize industry and maize dependent communities worldwide. The knowledge gained from this study is expected to provide the maize industry with genetic tools and knowledge for development of novel environmentally

friendly aflatoxin-resistant germplasm as well as elucidating oxylipin-mediated cross-kingdom communication between plants and fungi, which is applicable to other plant-organism interactions.

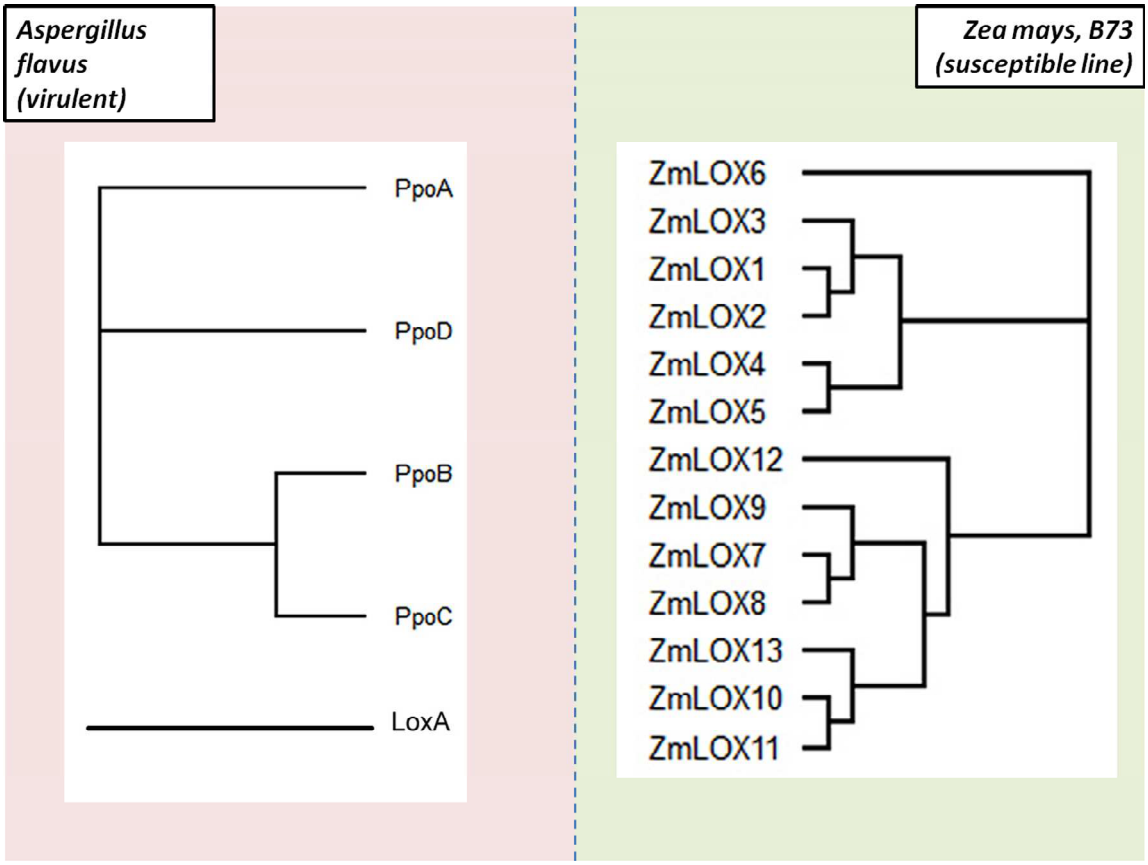


Fig. 1. Cladograms depicting oxygenases of *Aspergillus flavus* and maize organized by amino acid sequences. (Left) The *A. flavus* strain NRRL3357 genome houses four Psi producing oxygenases (Ppo) genes and a single LoxA gene. (Right) The maize B73 inbred line houses thirteen members are largely divided into two main groups: the 9-LOXs (LOX1, 2, 3, 4, 5, 12), and the 13-LOXs (LOX7, 8, 9, 10, 11, 13), LOX6 is an atypical isoform that possesses an unusual hydroperoxide lyase activity (Gao et al. 2008).

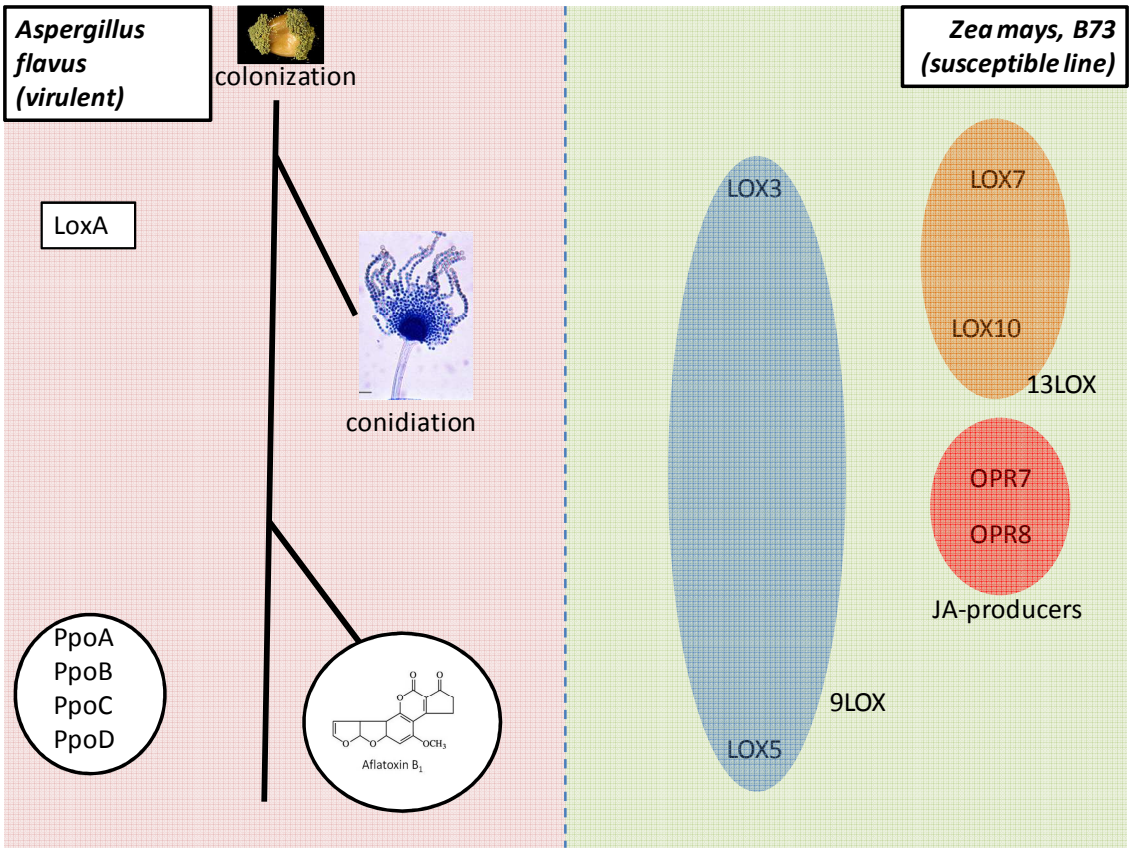


Fig. 2. Oxygenases of *A. flavus* (right) and maize (left) characterized for effect on fungal colonization, conidiation, and aflatoxin accumulation in maize seed within this study. The Ppo genes and a lone Lox gene of *A. flavus* as well as the maize 9-LOX, 13-LOX, and JA-producing OPR genes were investigated by using knockout or knockdown mutants and their isogenic or near-isogenic lines.

CHAPTER II

MATERIALS AND METHODS

PLANT AND FUNGAL MATERIAL

For the screening of the JA-perturbed mutants, maize wild type (B73 inbred line) and near-isogenic *LOX3* (*lox3-4* allele) (Gao et al., 2008b), *LOX5* (*lox5-3* allele) (Park, 2011), *LOX7* (*lox7-3* allele), *LOX10* (*lox10-3* allele) (Christensen et al., 2013), *OPR7* (*opr7-5* allele), *and OPR8* (*opr8-2* allele) (Yan et al., 2012) mutant seeds in the B73 genetic background were obtained from season 2013 in College Station, TX. Mutants of *lox3*, *lox5*, *lox7,* and *lox10* were advanced at least to the BC7F4 genetic stage. Mutants of *opr7* and *opr8* were at the BC6F6 genetic stage. For the study of *LOX3* with fungal oxygenases, maize wild type and near-isogenic *LOX3* (*lox3-4* allele) mutant seeds in the B73 genetic background were obtained from season 2009 in College Station, TX, which were at BC5F4 genetic stage. *Aspergillus flavus* (teleomorph, *Petromyces flavus*) strain NRRL3357 was used as wild type, and knockout and knockdown mutants were generated previously in this background (Table 1, (Horowitz Brown et al., 2008; Brown et al., 2009). Fungal strains were cultured at 28°C on potato dextrose agar (PDA) for 1-2 weeks.

KERNEL BIOASSAY

Kernel bioassays were performed with slight modifications following the protocol from (Christensen et al., 2012). Seeds were surface sterilized by shaking under 70% ethanol (v/v) for 5 min, sterile water for 1 min, 0.06% sodium hypochlorite (v/v) for 10 min, followed by rinsing with sterile water three times for 5 min each. Afterwards, autoclaved paper towels were used to pat kernels dry. Following inoculation, kernels were oriented embryo-side up and positioned out of contact with one another inside 20 ml glass scintillation vials, which were then placed inside humidity chambers. Humidity chambers were composed of clear plastic boxes lined with five paper towels saturated with 100 ml of sterile water and covered with Press'n Seal (Glad, Oakland, CA). Five small incisions in the covering were created to facilitate air exchange. Afterwards, the humidity chambers were placed inside an incubator connected to a continuous vacuum line with minor suction to draw out excessive volatiles that accumulate during the time-course. Incubation for the kernel assays began during the dark phase of a 12/12 dark/light cycle. After designated time-points, scintillation vials for conidia enumeration and secondary metabolites analysis were collected and placed into -80°C until processing. Tissue for expression analysis was immediately frozen in liquid N_2 and stored at -80°C until RNA isolation and quantification.

This study's designation	Strain	Genotype	Source
WT	NRRL3357	Wild type	He et al., 2007
A	TSHB3.1.19	*ΔppoA: pyrG; pyrG-*	Brown Horowitz et al., 2008
C	TRAW2.2.15	*ΔppoC: pyrG; pyrG-*	Brown Horowitz et al., 2009
D	TJBS4.1.85	*ΔppoA: pyrG; pyrG-*	Brown Horowitz et al., 2009
L	TSHB2.39	*ΔAflox: pyrG; pyrG-*	Brown Horowitz et al., 2008
ABD	TJBS5.1.60	*ΔppoD:pyrG;niaD:ppoA, ppoB* IRT:*niaD*	Brown Horowitz et al., 2009
ABCD	TSHB7.1	*ΔppoD:pyrG; niaD:ppoA, ppoB* IRT:*niaD ppoC:pyrG-*	Brown Horowitz et al., 2009
ABDL	TSHB6.3	*ΔppoD:pyrG; niaD:ppoA, ppoB* IRT:*niaD lox:pyrG*	Brown Horowitz et al., 2009
ABCDL	TSHB5.57	*ΔppoD:pyrG; niaD:ppoA, ppoB* IRT:*niaD ppoC, lox* IRT:phleomycin	Brown Horowitz et al., 2009

Table 1. Fungal mutants used in this study.

CONIDIA ENUMERATION, ERGOSTEROL QUANTIFIATION, AND AFLATOXIN B1 DETERMINATION

Conidia suspensions were prepared by vortexing kernels with 2.5 ml of methanol and immediately combining 50 µl of suspension with 50 µl of water. Next, diluted suspensions were enumerated using a hemocytometer.

Ergosterol extractions were prepared by incubating kernels with 5 ml of chloroform mixed with the 2.5 ml of methanol to produce 2:1 chloroform: methanol (v/v) solvent. Kernels in the extraction solution were incubated for 16 hours in the dark before being filtered through a 0.2 µm nylon-membrane pore syringe filter. Two µl of filtrate were combined with 50 µl of 20 µM C^{13}-cholesterol (cholesterol-25, 26, 27-^{13}C; Sigma cat. # 3707678) in methanol as internal standard and dried under a nitrogen stream. Afterwards, samples were resuspended in 100 µl of methanol and analyzed through liquid chromatography-mass spectrometry utilizing atmospheric photochemical ionization ((+)-APCI-MS/MS) (Headley et al., 2002). The system used an Ascentis Express C-18 Column (3 cm X 2.1 mm, 2.7 um) connected to an API 3200 LC/MS/MS with multiple reaction mentoring (MRM). The injection volume was two µl and the isocratic mobile phase consisted of methanol at a flow rate of 200 µl/min.

AF was quantified using the same methanol/ chloroform extractions as described above, through gradient liquid chromatography coupled with electrospray ionization ((-)-ESI-MS/MS). The system employed the column and mass spectrometer described above. AF transition ions used for quantification were m/z 313 to 241, 269, and 241 (Varga et

al., 2013). The injection volume was 2 µl and 300 µl/ min mobile phase consisted of solution A (10% methanol, 89% water, and 1% acetic acid) and solution B (97% methanol, 2% water, and 1% acetic acid) with the following gradient (time-% B solution): 3-15%, 5-100%, 8-100%, 9-15%, 12-15%, and 12.1-stop run). A five-point standard curve against a pure standard (aflatoxin B1, Cayman Chemicals cat# 11293) was used for quantification.

PHYTOHORMONE ANALYSIS

Phytohormone extraction followed (Christensen et al., 2013) with modifications: 500 µl of phytohormone extraction buffer (1-propanol/water/ HCl [2:1:0.002 v/v/v]) and 10 µl [5 uM solution] of deuterated internal standards: d-ABA ([$^2H_6$](+)-cis,trans-absisic acid; Olchemlm cat# 034 2721), d-IAA([$^2H_5$] indole-3- acetic acid; Olchemlm cat# 0311531, and d-JA (2,4,4-d_3; acetyl-2,2-d_2 jasmonic acid; CDN Isotopes cat# D-6936) and d-SA (d6- salicylic acid; Sigma cat#616796) was added to 100 ± 10 mg of ground infected kernel tissue. Samples were agitated for 30 min at 4°C under darkness, and then 500 µl of dichloromethane was added to samples and agitated for 30 min at 4°C under darkness. Samples were centrifuged at 13,000 RPM for 5 min and lower layer was collected into glass vial for evaporation under nitrogen gas stream. Samples were resuspended in 150 µl of methanol and centrifuged in 1.5 ml microcentrifuge tube at 14,000 for 2 min to pellet any debris. One hundred µl of supernatant was collected into

autosampler vial for direct injection into LC- (-)-ESI-MS/MS. The simultaneous detection of several hormones utilized methods by (Muller and Munne-Bosch, 2011) with modifications. The quantification utilized the same column and detector as above with a 600 µl/min mobile phase consisting of Solution A (0.05% acetic acid in water) and Solution B (0.05% acetic acid in acetonitrile) with a gradient consisting of (time-%B): 0.3- 1%, 2- 45%, 5-100%, 8-100%, 9-1%, 11-stop.

RNA ISOLATION AND QUANTIFICATION

Kernels were ground under liquid nitrogen and total RNA from kernel bioassays was extracted with kernel extraction buffer (50mM TRIS pH 8.0, 150 mM LiCl, 5mM EDTA pH 8.0, 1% SDS) for high polysaccharide rich tissue. Two hundred µl of kernel extraction buffer was combined with 200 µg of ground seed tissue and 200 µl phenol: chloroform (pH 4.3). The mixture was vortexed until thawed and incubated on ice for 5 min. The mixture was transferred into PHASELOCK tubes (Fisher, cat#FP2302810) for phase-separation based purification and centrifuged at 10,000 RPM for 5 min. 200 µl phenol: chloroform (pH 4.3) was added, mixed by vortex, and mixture was incubated on ice for 5 min, and centrifuged for 5 min at 10,000 RPM. Afterwards, RNA was purified from the aqueous phase over a column (Qiagen, cat#74104) according to manufacturer's specifications and quantified by spectroscopy (Nanodrop, Cole Palmer). Five µg of total RNA was DNAse treated (Thermo Scientific, cat# FEREN0521) and diluted to 2 ng/µl

for quantitative PCR (0.25 µl Verso enzyme mix, 12.5 µl 2X 1-Step qPCR SYBR Mix, 1.25 µl RT Enhancer, 2 µl 1 µM each forward + reverse primer, 1 µl 12.5 µM ROX, 4.5 µl water, and 2 µl of 4 ng/µl template; Thermo Scientific, cat# AB4100A) (Constantino et al., 2013).

DATA ANALYSIS

JMP 10.0.1 (SAS Institute, Inc) statistical package was used for data analysis. First, we analyzed the correlation between conidia suspensions, colonization and AF, as measured by methanol/ chloroform extractions. In order to meet the requirements of the respective statistical tests, data were normalized by taking the logarithm of conidia and the square root of colonization and AF. Normalized data were used to compute the Pearson correlation values and the corresponding p-values for various groupings of the samples based on either host or fungal genotypes. Significantly, ($p < 0.05$) correlated pairs in different genotype groups are given as a ranked list in the Table S2. We used regression analysis to compare between maize genotypes for statistically significant correlations.

The effects of maize genotype, fungal genotype, days post inoculation (dpi) within maize genotype, and maize x fungus genotypes on fungal parameters were examined via ANOVA (Table S2). Specifically, ANOVA examined the effects of maize genotype, fungal genotype, maize x fungal genotype, and dpi within maize genotype on

each of the three fungal parameters, i.e. colonization, conidiation, and AF accumulation. Because the effects of (maize x fungal genotype, fungal genotype, and dpi within maize genotype) were variably significant among the fungal parameters (see *Results*), subsequent Tukey's HSD compared each fungal parameter between fungal strains and maize genotype at 3 and 5 dpi. In particular, these ANOVAs examined the effects of fungal genotype and maize genotype; changes in fungal parameters (i.e. increase or decrease) between 3 and 5 dpi within each of the four fungal x maize genotype combinations tested via Student's *t*-tests with Bonferroni multiple comparisons correction.

Correlations between fungal parameters and phytohormones and their ratios were performed with Spearman's rank correlation coefficient (also known as, Spearman's rho) nonparametric test utilizing means of variables.

CHAPTER III

JA-PERTURBED LOX AND OPR MUTANTS DISPLAY ALTERED LEVELS OF RESISTANCE TO SEED COLONIZATION BY *A. FLAVUS*

INTRODUCTION

The linolenic acid-derived phytohormones known as jasmonates possess diverse signaling roles during plant responses to environmental stress and developmental stimuli (Wasternack, 2007; Wasternack and Hause, 2013). The jasmonate group is composed of jasmonic acid (JA), its precursors, and various derivatives. Biosynthesis of JA initiates in the chloroplast with the cleavage of linolenic acid (C18:3) from the plastid membrane by lipases followed by peroxidation by 13-lipoxygenase (13-LOX). The resulting hydroperoxide, 13-hydroperoxy octadecatrienoic acid (13*S*-HPOTE), provides substrate to 13-allene oxide synthase (13-AOS) marking the first committed reaction for JA biosynthesis. The allene is cyclized into cis-(+)-12-oxo-phytodienoicacid (OPDA) by allene oxide cyclase (AOC). OPDA is subsequently exported into the peroxisome for reduction by OPDA- reductase (OPR) and undergoes three rounds of β-oxidation producing (+)-7-iso-jasmonic acid (Feussner and Wasternack, 2002).

Aspergillus flavus (teleomorph, *Petromyces flavus*) (Horn et al., 2009) is a soil-borne fungal saprophyte that can infect and colonize oil-rich crops where it contaminates seed with secondary metabolites known as mycotoxins. One of the most hazardous

mycotoxins is aflatoxin, described as the most carcinogenic naturally occurring compound known. Consumption of aflatoxin by animals increases potential for hepatic cancer and toxicity (Bennett and Klich, 2003). In efforts to protect human and animal health, many nations have implemented regulations to limit acceptable contamination levels in corn, milk, etc. (Richard et al., 2003). Naturally, this renders an allotment of yield unfit for human or animal consumption contributing to significant economic losses worldwide. Many strategies are currently underway to control aflatoxin contamination, which includes breeding for increased aflatoxin resistance, biocontrol, insect control, and antifungal resistance (Cleveland et al., 2003).

Previous studies offer limited insight to understand the role of JA during *A. flavus* infection. *In vitro* treatment with exogenous methyl-JA (MeJA) has demonstrated an ability of *Aspergilli* to respond to JA treatment, albeit, in a species- or treatment-specific fashion. When applied as a volatile to *A. flavus* colonies, MeJA, suppressed aflatoxin production (Goodrich-Tanrikulu et al., 1995), however when applied directly to liquid media of cultured *A. parasiticus*, MeJA induced aflatoxin accumulation (Vergopoulou et al., 2001). Although these studies were in different laboratories and likely under different conditions, these studies suggest a species-specific response following JA perception as well as a direct effect of JA in *A. flavus* secondary metabolism regulation.

Recent analyses of maize *lox3-4* mutants provided additional correlative evidence for the importance of JA in maize-*A. flavus* interactions. The LOX3 knockout mutant, *lox3-4*, was found to be highly susceptible to *Aspergilli (Gao et al., 2009)*. In

kernel bioassays, *lox3-4* mutant kernels supported increased visual fungal colonization, conidia, and aflatoxin production compared with the near-isogenic WT. In agreement, field trials during two years and across two locations showed that *lox3-4* accumulate significantly more aflatoxin than WT. JA accumulation was increased in *lox3-4* mutant kernels after *A. flavus* infection compared to WT, suggesting that JA may contribute to its increased susceptibility. The study described in this chapter aimed to elucidate a specific role of maize JA biosynthetic and signaling genes during seed infection by *A. flavus* on fungal pathogenicity processes including colonization, conidiation, and aflatoxin accumulation. To understand the biochemical basis behind any changes identified in the mutants as compared to WT, we have measure a set of plant hormones during the infection process.

RESULTS

Response of maize JA-perturbed mutants to *Aspergillus flavus* infection

To investigate comprehensively the role of JA during maize seed infection by *Aspergillus flavus*, near-isogenic knockout mutants of JA biosynthetic genes and other genes known to regulate JA biosynthesis were utilized in kernel bioassays. All mutants used in the study were backcrossed seven times to B73 inbred line. B73 was chosen due to its susceptibility to *Aspergillus* ear rot, wide use in corn breeding programs, and

availability of the genome sequence. Mutants employed for this study were the putative JA-producing 13-LOX, *lox7*, and the mutants in the JA-producing OPR paralogs, *opr7* and *opr8* (Yan et al. 2012), as well as, *lox5* (Park 2012) and *lox10* (Nemchenko et al. 2013) mutants, both of which produce lower JA levels in wounded leaves. The pathogen strain chosen for this study was *A. flavus* NRRL3357, the wild type strain that produces high levels of aflatoxin, and the genome of which has been recently sequenced.

Colonization, conidiation, and aflatoxin accumulation

At three and five days following *A. flavus* inoculation, samples were collected to assess colonization, conidiation, and aflatoxin accumulation. Overall, both *lox3* and *lox7* mutant kernels were colonized more extensively by *A. flavus* compared to WT ($p < 0.0001$ for both) and this pattern was observed at both time-points (Fig. 3). No other maize genotype displayed statistically significant difference from WT, which suggests that intact LOX3 and LOX7 specifically function positively in seed defense against *A. flavus* fungal colonization.

In terms of conidiation, when normalized for fungal biomass, *lox10* mutants supported a three-fold increased level of conidiation compared to WT ($p = 0.0071$) at 3 days post infection (dpi) (Fig. 4). However, conidiation levels were comparable to WT by 5 dpi. In contrast to *lox10*, *lox7* mutants supported levels similar to WT at 3 dpi, yet at 5 dpi, they were unable to support the WT level of conidiation and instead displayed a

three-fold decrease ($p < 0.0001$). All other maize genotypes supported conidiation levels that were not statistically different from WT at either three or 5 dpi. Taken together, this suggests that LOX10 negatively regulates early conidiation, while LOX7 positively regulates late conidiation.

Concerning aflatoxin accumulation normalized to fungal biomass (as measured by content of fungus-specific membrane lipid, ergosterol), almost all maize genotypes supported decreased aflatoxin levels (Fig. 5) at both 3 and 5 dpi. At 3 dpi, *lox7*, *lox10*, *opr7*, and *opr8* accumulated decreased levels compared to WT kernels. While not statistically significant, *lox5* supported a decreased level of aflatoxin accumulation at 3 dpi. At 5 dpi, *lox5*, *lox7*, *lox10*, *opr7*, and *opr8* accumulated decreased levels compared with WT. Unexpectedly, more heavily colonized *lox3* mutants did not display any increase in the levels of aflatoxin when normalized for fungal biomass at either three or 5 dpi. Taken together, this suggests that all tested JA-perturbed mutants supported decreased levels of aflatoxin accumulation compared to WT.

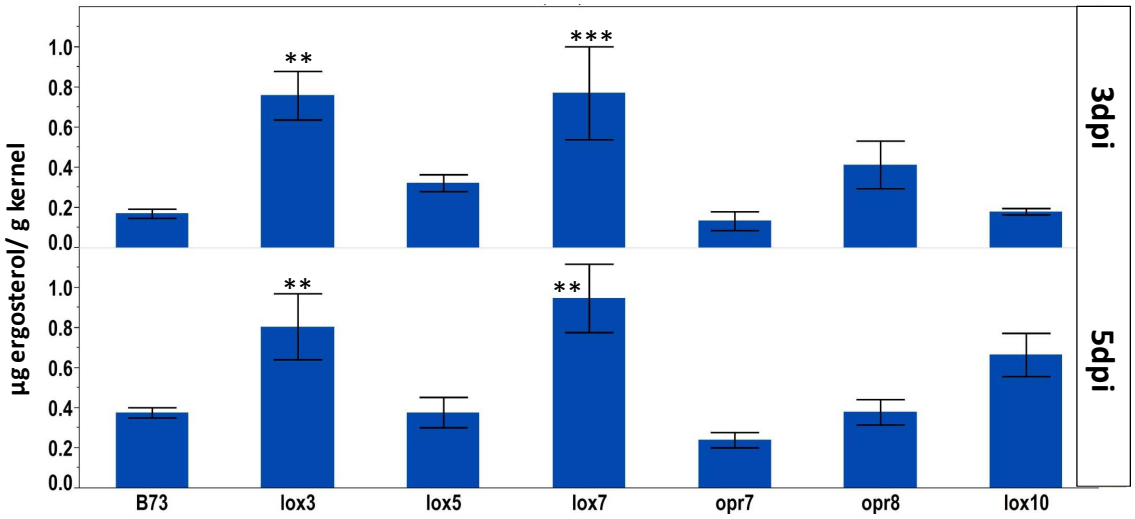


Fig. 3. *A. flavus* colonization of maize WT and the mutants perturbed in JA biosynthesis, *lox3*, *lox5*, *lox7*, *lox10*, *opr7*, and *opr8*. The Y-axis represents ergosterol content (µg / g kernel) and X-axis represents maize genotypes. Top and bottom halves of the graph represents 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant difference from WT (Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

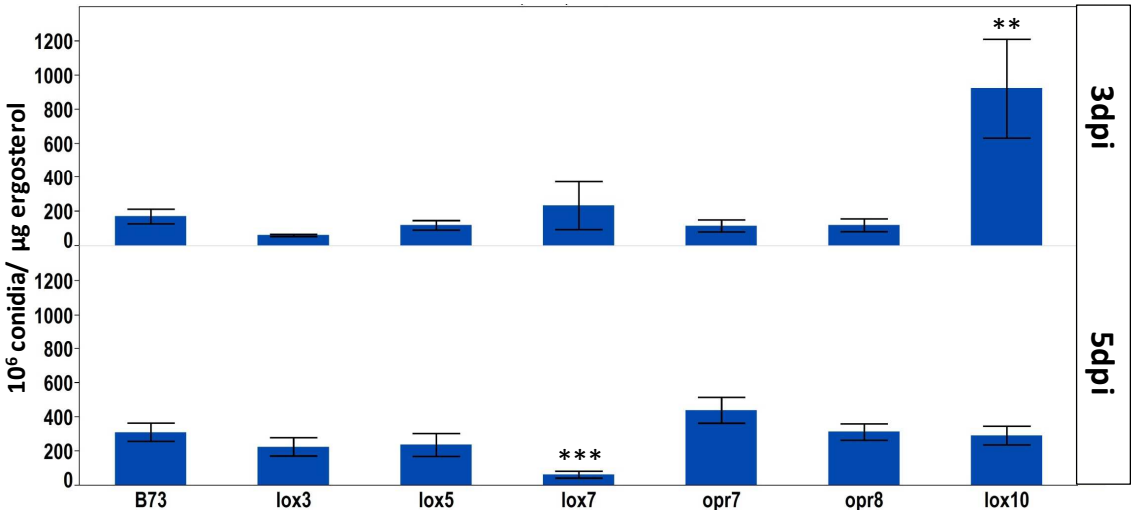


Fig. 4. *A. flavus* conidiation on maize WT and the mutants perturbed in JA biosynthesis, *lox3*, *lox5*, *lox7*, *lox10*, *opr7*, and *opr8*. The Y-axis represents fungal-biomass dependent conidiation (10^6 spores/ µg ergosterol) and X-axis represents maize genotype. Top and bottom halves of the graph respectively represents 3 and 5 days post inoculation (dpi). Bars are means ± SE; n=5. Asterisks represent statistically significant difference from WT (Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

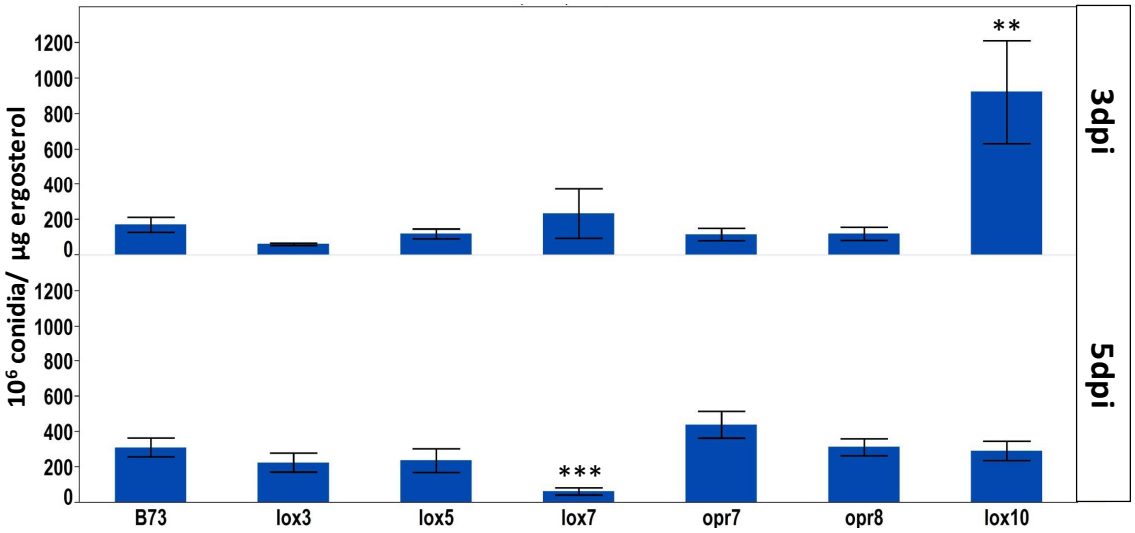


Fig. 5. Aflatoxin B_1 accumulation in maize WT and mutants perturbed in JA biosynthesis, *lox3*, *lox5*, *lox7*, *lox10*, *opr7*, and *opr8*. The Y-axis represents fungal-biomass dependent aflatoxin accumulation (µg aflatoxin/ µg ergosterol) and X-axis represents maize genotypes. Insert in top half of the graph represents 3 days post inoculation (dpi), and the bottom half of graph respectively represents 5 dpi, respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant difference from WT (Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

Phytohormone content

To determine the effect of JA-perturbed mutants on phytohormone content following infection by *A. flavus*, abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA), and JA-isoleucine conjugate (JA-Ile) levels were quantified at 3 and 5 dpi in inoculated and mock treated control kernels. Statistically, the main effect of time after inoculation was observed for ABA, JA, and JA-Ile ($p < 0.0001$ for all); and maize genotype had the greatest effect on ABA, IAA, and JA-Ile ($p = 0.0001$, 0.0001, and 0.013, respectively); and the strongest effect of infection was found for the content of IAA, JA, and JA-Ile ($p = 0.0001$, 0.0008, and 0.0454, respectively).

The terpenoid-derived hormone ABA showed similar levels in most genotypes and treatments at 3 dpi. At 3 dpi, mock-treated *lox5* mutant kernels accumulated less ABA compared with mock-treated B73 ($p = 0.01$) (Fig. 6). At 5 dpi, mock treated kernels tended to accumulated 100% increased levels of ABA compared to their mock-treated components and this was most pronounced with *lox7*, and *lox10* genotypes. Remarkably, at 5 dpi, *lox3* mutants lost this pattern so that ABA levels in control and inoculated kernels are statistically higher and WT and indistinguishable from each other. Taken together, the results obtained for most mutants and WT suggest that *A. flavus* infection of maize seed induces ABA accumulation during more advanced disease stages, and LOX3 negatively regulates both germination- and infection-related ABA accumulation. Additionally, LOX5 appears to positively regulate early-germination-related ABA accumulation.

The tryptophan-derived phytohormone, IAA, was reduced nine-fold in inoculated seeds compared with controls for nearly all genotypes tested, with the exception of *opr7* at 3 dpi (Fig. 7). Additionally, several knockout mutants displayed aberrant IAA accumulation in either inoculated or mock-treated seeds compared to WT. At 3 dpi, mock-treated *lox5*, *opr7*, and *opr8* accumulated decreased IAA levels compared with mock B73 and this trend continued into 5 dpi for *opr7* and *opr8* mock-treated seed. In contrast to the mock controls, at 3 dpi, inoculated seed of *lox3*, *opr7*, and *opr8* accumulated increased levels of IAA compared with WT by eight- and two- and two-folds, respectively. At 5 dpi, only *lox7* showed reduced IAA accumulation in inoculated seed compared to WT for all genotypes tested. These results suggest that *A. flavus* infection of maize seed suppresses IAA accumulation at early and advanced diseases stages. At early disease stages, LOX3, OPR7, and OPR8 appear required to suppress infection-induced IAA accumulation. However, at advanced disease stages, LOX7 appears required for normal IAA production.

The fatty acid-derived phytohormone JA displayed a general trend of increased accumulation at three days in inoculated seeds compared with the mocks for most genotypes tested at 3 dpi (Fig. 8). However, this trend reversed at 5 dpi so that inoculated seeds accumulated decreased JA compared with mock-treated seeds. These trends were most evident with *lox3* and *lox7* at 3 dpi, and for *lox3*, *lox7*, *lox10*, and *opr7* at 5 dpi. At 3 dpi, mock treated *lox3*, *lox7*, and *lox10* accumulated decreased levels of JA compared to mock-treated WT. Additionally, while *lox7* showed the same tendency, only *lox3* at 3 dpi, accumulated statistically significantly higher levels of JA compared

with WT in inoculated seeds. These results suggest during *A. flavus* infection, maize seeds accumulate increased JA at early disease stages, but decreased levels at the advanced colonization stages. LOX3, LOX7, and LOX10 appear to regulate positively early germination-related JA accumulation. During *A. flavus* infection, LOX3 and LOX7 appear to negatively regulate JA accumulation in seed.

The biologically active amino acid derivative of JA, JA-Ile, displayed a general trend of increased accumulation in inoculated seeds compared with mock-treated seeds for all genotypes at 3 dpi (Fig. 9). This increase in JA-Ile was most pronounced in *lox5, lox10,* and *opr7* seeds. At 5 dpi, only B73 seeds showed a statistically significant decrease in JA-Ile accumulation in inoculated seeds compared to mocks. In contrast, inoculated *lox5* seeds accumulated increased JA-Ile in inoculated seeds compared to mock controls. At 3 dpi, mock-treated kernels of *lox5, opr7,* and *opr8* accumulated decreased JA-Ile as compared to mock-treated WT at 3 dpi. These results suggest *A. flavus* infection induces JA-Ile accumulation in maize seed at early disease stages but suppresses JA-Ile accumulation at later disease stages. LOX5 and LOX10 appear to be required to suppress JA-Ile accumulation during advancement of disease progression.

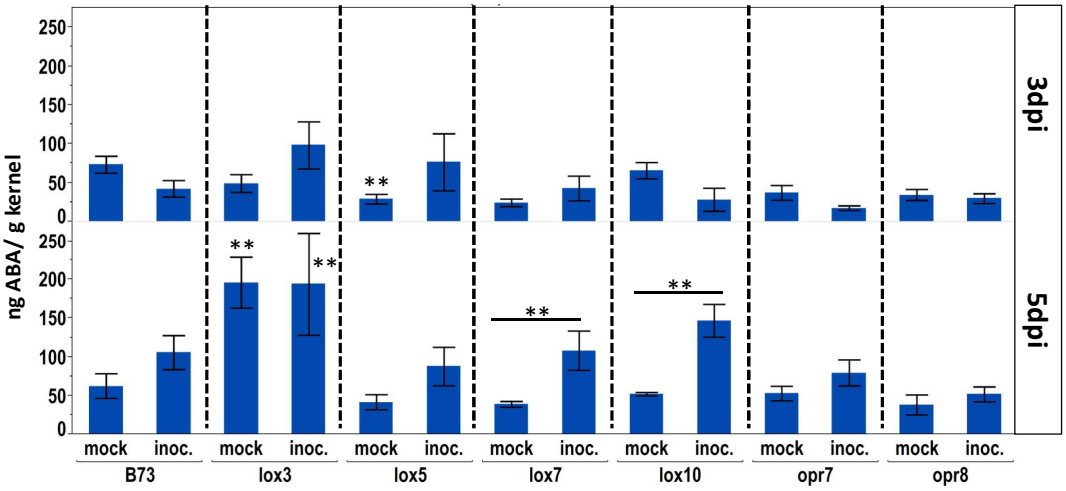


Fig. 6. ABA accumulation in *Aspergillus flavus* inoculated and mock-treated kernels of maize WT and the mutants perturbed in JA biosynthesis. The Y-axis represents ABA accumulation (ng ABA/ g kernel) and X-axis represents maize genotype. The top half of the graph represents three days post inoculation (dpi) and the bottom half of graph represents 5 dpi, respectively. Bars are means ± SE; n=five. Asterisks represent statistically significant difference from WT inoculated or mock-treated kernels (Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$), Asterisks over bars represent statistically significant difference between inoculated and mock-treated kernel (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

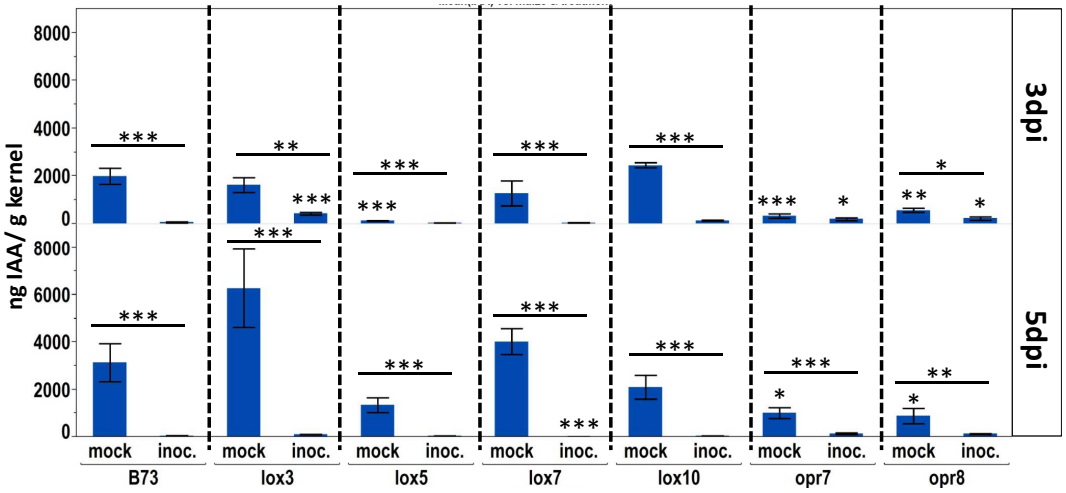


Fig. 7. IAA accumulation in *Aspergillus flavus* inoculated and mock-treated kernels of maize WT and the mutants perturbed in JA biosynthesis. The Y-axis represents ABA accumulation (ng IAA/ g kernel) and the X-axis represents maize genotype. The top half of graph represents three days post inoculation (dpi) and the bottom half of graph represents 5 dpi, respectively. Bars are means ± SE; n=five. Asterisks represent statistically significant difference from WT inoculated or mock-treated kernels (Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$), Asterisks over bars represent statistically significant difference between inoculated and mock-treated kernel (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

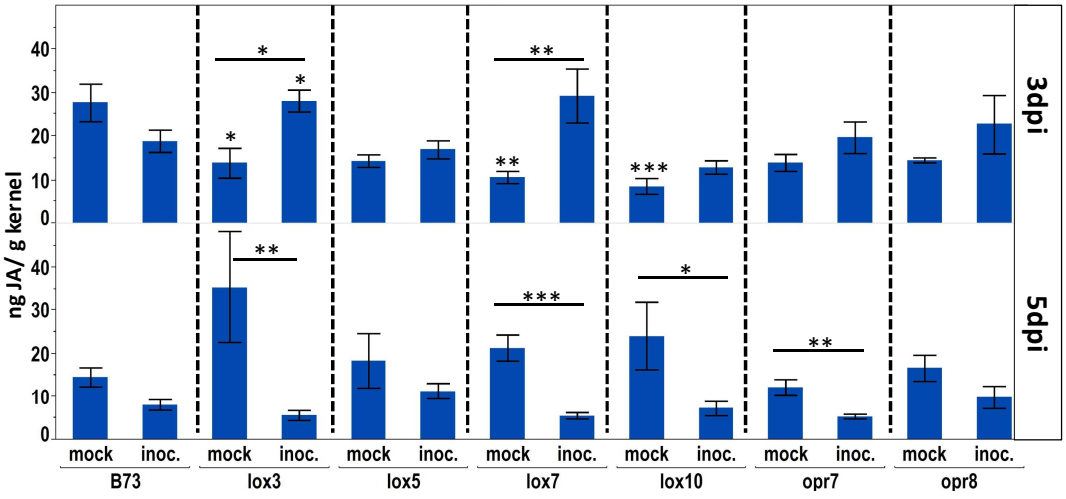


Fig. 8. JA accumulation in *Aspergillus flavus* inoculated and mock-treated kernels of maize WT and mutants perturbed in JA biosynthesis. The Y-axis represents ABA accumulation (ng JA/ g kernel) and the X-axis represents maize genotype. The top half of the graph represents three days post inoculation (dpi) and the bottom half of graph represents 5 dpi, respectively. Bars are means ± SE; n=five. Asterisks represent statistically significant difference from WT inoculated or mock-treated kernels (Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$), Asterisks over bars represent statistically significant difference between inoculated and mock-treated kernel (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

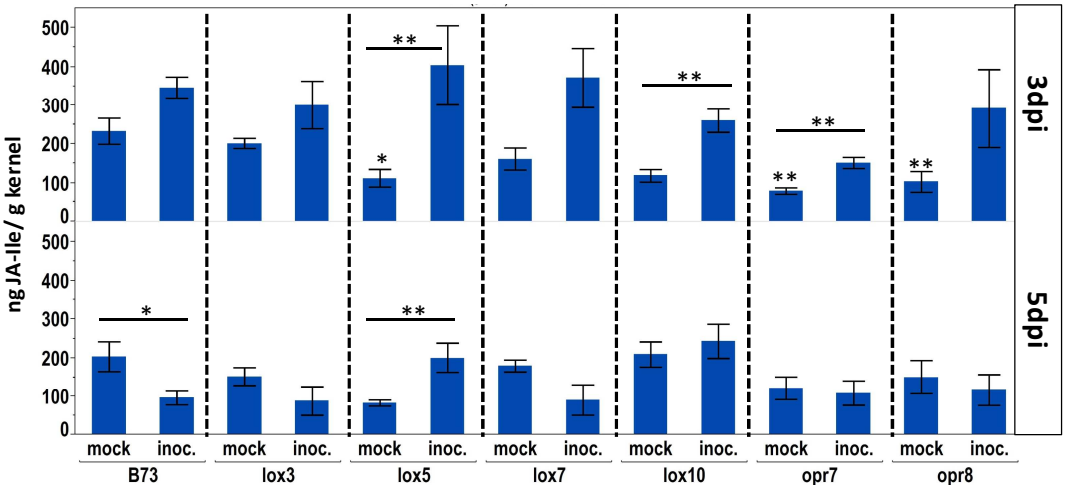


Fig. 9. JA-Ile accumulation in *Aspergillus flavus* inoculated and mock-treated kernels of maize WT and mutants perturbed in JA biosynthesis. The Y-axis represents ABA accumulation (ng JA-Ile/ g kernel) and the X-axis represents maize genotype. The top half of the graph represents three days post inoculation (dpi) and the bottom half of graph represents 5 dpi, respectively. Bars are means ± SE; n=five. Asterisks represent statistically significant difference from WT inoculated or mock-treated kernels (Dunnett's test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$), Asterisks over bars represent statistically significant difference between inoculated and mock-treated kernel (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

Variable	by Variable	Spearman ρ	Prob>|ρ|
colonization	ABA	0.67	0.009
aflatoxin	ABA/ JA	0.81	0.004
aflatoxin	JA	-0.76	0.002
aflatoxin	JA-Ile	-0.74	0.003
aflatoxin	ABA	0.67	0.009

Table 2. Spearman's correlation of hormone ratios with fungal parameters of maize WT and JA biosynthesis mutants, *lox3*, *lox5*, *lox7*, *lox10*, *opr7*, and *opr8* kernels infected by *A. flavus* WT.

Phytohormone correlations

Correlation analysis was conducted to determine any association between phytohormone content, ratios amongst phytohormones, and the fungal parameters of colonization, conidiation, and aflatoxin accumulation (significant correlations shown in Table 2). In this data set, ABA positively correlated with colonization (rho = 0.67, p = 0.009), and aflatoxin (rho = 0.67, p = 0.009). Aflatoxin negatively correlated with JA (rho = -0.76, p = 0.002) and JA-Ile (rho = -0.74, p = 0.003). The ratio of ABA/ JA correlated positively with aflatoxin accumulation (rho = 0.81, p = 0.004).

DISCUSSION

Despite advancements in chemical and breeding technologies, the maize industry remains threatened by mycotoxigenic fungi such as *Aspergillus flavus.* Unfortunately, little is known of the molecular mechanism during maize seed defense against *A. flavus*.

Phytohormones function as signals during defense against biotic stress and are known to either, induce a resistant response, or to promote susceptibility. In this study, compared with mock-treated seeds, inoculated seeds increased ABA but decreased IAA accumulation throughout the disease progression. However, at early disease stages, JA and JA-Ile increased accumulation in inoculated seed compared with mock treated seed, but this was reversed with disease progression.

This study provides genetic evidence that implicate specific maize LOX and OPR gene family members in the regulation of colonization, conidiation, and aflatoxin accumulation (Fig. 11). In terms of colonization, both *lox3* and *lox7*, a 9- and 13-LOX, respectively, positively contribute to defense against colonization at both three and 5 dpi. Despite belonging to separate LOX branches, it is likely that their down-stream product converge on the same biochemical or molecular targets. Previously, it was shown that *lox3* mutant roots constitutively over-expressed JA biosynthetic genes in roots (specifically, LOX8, AOS, AOC, and OPR8) (Gao et al., 2008b), suggesting a role of LOX3 is to suppress the JA pathway. LOX7 is the closest paralogs to LOX8 and during *A. flavus* infection may compete for the substrate with LOX8 and/or other JA-producing LOXs. While only *lox3* showed statistically significantly increase in JA levels in inoculated kernels compared to WT, *lox7* showed a similar pattern that was not statistically supported at the $p < 0.05$ cutoff level. Yet, both *lox3* and *lox7* displayed statistically significantly higher levels of JA in inoculated kernels compared with mock-treated kernels. No other genotype displayed this increase at 3 dpi, and all genotypes reduced JA content by 5 dpi. Since both *lox3* and *lox7* mutants are more susceptible to colonization by *A. flavus*, this evidence strengthens the notion of JA as a susceptibility factor during early disease progression. Since for many biotrophic or hemibiotrophic pathogens, one pathogenicity strategy is to increase JA in the colonized tissue (Thaler et al., 2004) (presumably resulting in decreased SA, a defense hormone against these pathogens) it is tempting to speculate that *A. flavus* may have a hemibiotrophic pathogenic lifestyle. I hypothesize therefore, that during early disease stages JA

biosynthesis is upregulated to suppress defenses against biotrophic pathogens, but with progression of disease the pathogen transitions into a necrotrophic strategy. In contrast, both *lox3* and *lox7* mutants displayed aberrant IAA accumulation, yet *lox3* had increased IAA at 3 dpi while *lox7* had decreased IAA accumulation at 5 dpi, suggesting their down-stream signals also modulate hormone-crosstalk. Interestingly, both *LOX3* and *LOX7* are expressed in developing embryos (Fig. 10), at a time when naturally occurring infection by Aspergilli take place (Anderson et al., 1975), suggesting a defensive role during attack under a natural setting.

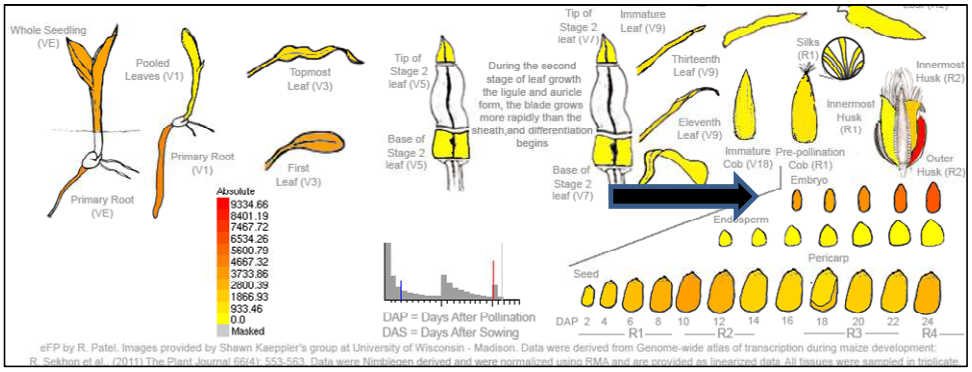
The 13-LOXs, LOX7 and LOX10, appear to regulate conidiation, although oppositely. A previous report identified LOX10 as the sole green leaf volatile- producing LOX isoform in maize leaves (Christensen et al., 2013), yet it remains to be determined if LOX10 also functions similarly in maize seed during germination or during fungal attack. However, this system employed a vacuum to remove excessive volatiles from the incubation chamber, which suggests non-volatile C-12-derived LOX10-products (e.g., traumatin and traumatic acid) are responsible (Kallenbach et al., 2011).

Most JA-biosynthesis and signaling mutants displayed decreased aflatoxin accumulation compared with WT and both three and 5 dpi, implicating JA-mediated signaling as a positive regulator of maize aflatoxin accumulation.

Interestingly, ABA correlated positively with increased fungal colonization, immediately suggesting drought as a factor in maize susceptibility to *A. flavus*. Currently, drought is recognized as an imparted stress on the plant indirectly favoring susceptibility (Klich, 2007), however this observation suggests drought induced ABA

accumulation may also promote susceptibility through signaling (Robert-Seilaniantz et al., 2011). While JA and JA-Ile negative correlated with aflatoxin accumulation, the ratio of ABA to JA positively correlated with aflatoxin accumulation suggesting the interaction of these signaling pathways determine aflatoxin contamination.

Zm*LOX3*



Zm*LOX7*

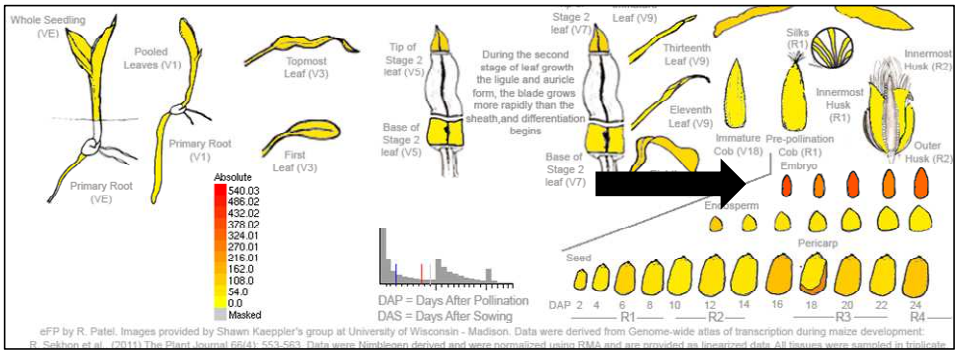


Fig. 10. Expression of ZmLox3 and ZmLox7 gene expression from the Maize eFP browser (Sekhon et al., 2011). Red highlighted regions show increased absolute transcript accumulation. Black arrows emphasize developing embryo expression levels.

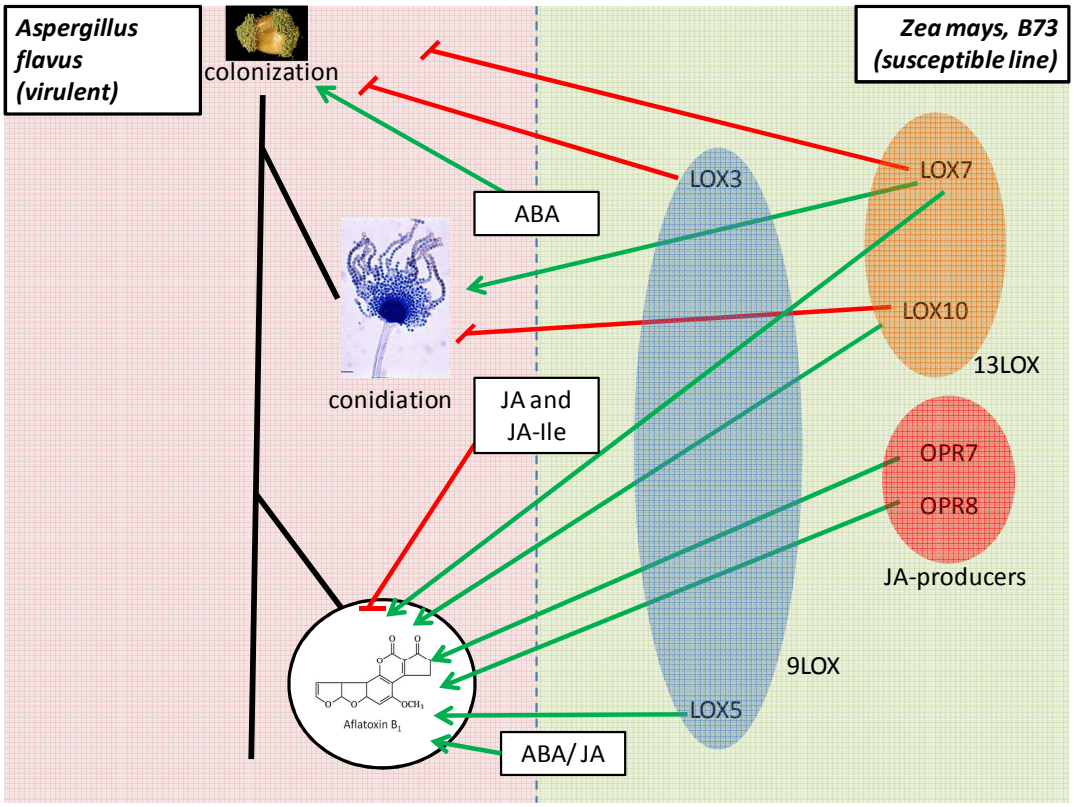


Fig. 11. Effect of maize JA biosynthesis genes with fungal process of colonization, conidiation, and aflatoxin accumulation of *A. flavus.* During disease development, LOX3 and LOX7 negatively regulate colonization. While LOX7 positively regulates conidiation, LOX10 negatively regulates conidiation. All the JA-biosynthesis genes and LOX5 positively promote aflatoxin accumulation. The phytohormone ABA positively correlated with colonization, and JA and JA-Ile negatively correlated with aflatoxin accumulation. The hormone ratio of ABA/ JA positively correlated with aflatoxin accumulation. Green lines indicate positive regulation and red lines indicate negative regulation.

CHAPTER IV

ROLE OF MAIZE LOX3 AND *ASPERGILLUS FLAVUS* OXYLIPIN BIOSYNTHESIS GENES IN SEED-PATHOGEN INTERACTIONS

INTRODUCTION

Seed-rotting fungi not only deteriorate seed quality through tissue maceration but also render the grains unfit for human or animal consumption through the production of toxic secondary metabolites that contaminate seed. Governments worldwide recognize these mycotoxins for their toxicity and carcinogenicity and actively regulate their concentrations in various commodities (Bennett and Klich, 2003; Yu et al., 2008). Seed containing mycotoxin concentrations above regulatory thresholds are destroyed, causing significant economic losses to their respective industries; however, in countries where the infrastructure cannot feasibly regulate mycotoxins, public health remains threatened. Unfortunately, many of these communities are located in areas where environmental conditions are conducive for infection to take place before and after harvest.

Aflatoxins produced by *Aspergillus spp.* are frequent mycotoxin contaminants of maize especially under drought or heat stress. Aflatoxin B_1 (AF) has been under extensive studies in mammalian systems to determine the mode of action for its toxic, teratogenic, and carcinogenic properties. Despite the well-understood extreme carcinogenic potency of AF, an explanation for the role of this metabolite in fungal

biology has escaped generations of research (Amaike and Keller, 2011). Following infection of maize seeds by *Aspergillus spp.*, many chemical signals from both the plant host and fungal pathogen govern disease development and secondary metabolite production. Recently, a class of oxygenated lipids, known as oxylipins, has gained appreciation as a major group of molecular signals that regulate colonization, sporulation, and mycotoxin production (Tsitsigiannis and Keller, 2007; Christensen and Kolomiets, 2011).

Oxylipins, produced through enzymatic and non-enzymatic activity in animals, fungi, and plants, are potent endogenous regulators of diverse metabolic and physiological processes (Andreou et al., 2009; Brodhun and Feussner, 2011). In plants, the lipoxygenase pathway is the major producer of over 500 distinct oxylipins; however, the function of the majority of them remain to be characterized (Feussner and Wasternack, 2002; Andreou et al., 2009). Undeniably, the best understood plant oxylipin are jasmonic acid, its derivatives, and precursors called jasmonates (JA). JA regulates development and adaptation to diverse biotic and abiotic stresses both positively or negatively depending on the specific stress stimuli (Wasternack, 2007; Wasternack and Hause, 2013). In *Aspergilli*, oxylipins are produced primarily through the activity of a class of dioxygenases known as Psi (precocious sexual inducer) producing oxygenases (Ppo). Diverse Ppo products act as molecular signals and regulate vegetative growth, sexual and asexual reproduction, virulence, secondary metabolism, and density-dependent production of spores and overwintering structures ((Horowitz Brown et al., 2008; Brodhun and Feussner, 2011) (Tsitsigiannis and Keller, 2007)). Oxylipins from

fungi and plants are biochemically and structurally similar prompting a novel hypothesis: during plant-fungal interactions, reciprocal exchanges of these compounds modulate the responses of opposing partner (Gao and Kolomiets, 2009; Christensen and Kolomiets, 2011; Borrego and Kolomiets, 2012). The maize-*Aspergillus* pathosystem provides an agro-economically valuable model to elucidate the potential oxylipin cross-talk, as both host and pathogen are readily amendable to genetic manipulation. In this study, we employed a maize lipoxygenase 3 mutant, from now called *lox3*, and a unique set of *A. flavus* oxylipin-mutants to discern interplay between the host and the pathogen oxylipins on fungal ability to colonize the host, produce spores and mycotoxin.

Previous research showed that mutation of maize *LOX3* gene results in significant increase in AF contamination levels under field conditions (Gao et al., 2009). Disruptions of the *Aspergillus flavus* genes encoding multiple Ppos (also called linoleate diol synthase) and a sole lipoxygenase gene, *LoxA*, have provided genetic evidence for the role of fungal oxylipins in growth, sporulation, quorum sensing, and mycotoxin biosynthesis (Tsitsigiannis et al., 2005; Horowitz Brown et al., 2008; Brown et al., 2009). This goal of this study was to test the hypothesis that plant and fungal oxylipins that are involved in intricate signal communication that govern whether the host is susceptible or resistant to colonization by the pathogen and the ability of the fungus to produce spores and mycotoxins.

In this study, we tested colonization, conidiation, and AF accumulation in the *lox3* mutant and near-isogenic wild-type kernels infected with *A. flavus* wild type and a diverse collection of oxylipin mutant *A. flavus* strains (Table 1) in kernel bioassays; the

single mutants of *ΔppoA, ΔppoC, ΔppoD, ΔloxA,* triple mutant in the *ΔppoA , ΔppoB,* and *ΔppoD,* quadruple mutants *ΔppoA, ΔppoB, ΔppoC, ΔppoD* and *ΔppoB, ΔppoD, ΔloxA,* and pentuple mutant *ppoA, B, C, D, loxA,* the latter disrupted in all predicted oxylipin biosynthesis genes. Throughout this manuscript for simplicity these mutant strains were designated as A, C, D, L, ACD, ABCD, and ABCDL (see Table 1). The phenotypic, molecular and biochemical analyses of these combinations of diverse mutants uncovered a complicated interaction between maize and fungal oxylipins, and their effect on the biosynthesis of diverse plant hormones.

RESULTS

Colonization, conidiation, and aflatoxin accumulation

Colonization levels were determined through ergosterol quantification by LC-MS/MS, conidia were enumerated by using a hemocytometer, and AF was measured by LC-MS/MS. Our analysis of fungal parameters utilized fungal biomass, determined by ergosterol quantification, to normalize conidiation and AF accumulation per unit of the fungal biomass. Examined this way, these parameters are relevant to fungal biology and plant-fungal interactions and relative shifts in secondary metabolism are detected allowing opportunity to appropriately implicate contributions of specific fungal genes on

fungal process. We report for the first time a method to assess simultaneously these three important disease parameters from a single sample.

The effects of maize genotype, fungal genotype and days post inoculation (dpi) within a maize genotype, and maize verses fungus genotypes on fungal parameters were examined by using ANOVA statistical analyses. Overall, maize genotype significantly affected colonization ($p < 0.01$) and conidiation ($p < 0.01$), fungal genotype significantly affected conidiation ($p < 0.01$) and AF accumulation ($p < 0.01$), and maize by fungal genotype combination significantly affected fungal conidiation ($p < 0.01$). In addition, dpi affected conidiation and AF accumulation significantly, though differently in the maize WT and *lox3* mutant genotypes. The effects of fungal genotype and maize genotype on each fungal parameter were examined separately for each fungal genotype in relation to WT fungus (i.e., fungal genotype/maize genotype affected colonization etc.)

Colonization

The most statistically significant effect of the host genotype was with colonization where the *lox3* mutant kernels supported increased colonization compared to WT kernels at 5 dpi as was seen before for both *A. nidulans* and *A. flavus* (Gao et al., 2009). Most *Aspergillus* mutants displayed a delayed colonization phenotype on WT kernels and this was most pronounced for strains L, ABCD, and ABCDL (Fig. 10),

however by 5 dpi these strains reached colonization levels comparable to WT *A. flavus*. Disruption of *LOX3* in the maize host alleviated the colonization delay in ABCD and ABCDL, but not in any other mutant strains except for L. At day 3, L strain was unable to colonize either WT or *lox3* mutant kernels as successfully as WT *A. flavus*.

Conidiation

Maize genotype, fungal genotype, and dpi all affected conidiation. Of all fungal pathogenicity processes, conidia production appears to be affected the most by both the host and the fungal genotypes and revealed contributions of the diverse oxylipin biosynthesis genes from both the host and the fungus. At 5 dpi, *lox3* mutants supported increased conidiation levels compared to those of WT kernels inoculated with A, D, and L strains, and this trend was similar for strain C (Fig. 11). Strains A and ABCD both had strong negative correlation between colonization and conidiation (respectively, rho = -0.99, -0.84 $p < 0.01$, <0.01, Table 2). However, at 5 dpi, strains ABD and ABCD did not display any increased conidiation on *lox3* mutant kernels compared to WT strain. Interestingly, deletion of functional *loxA* from ABD and ABCD partially restored the *LOX3*-dependent conidiation response.

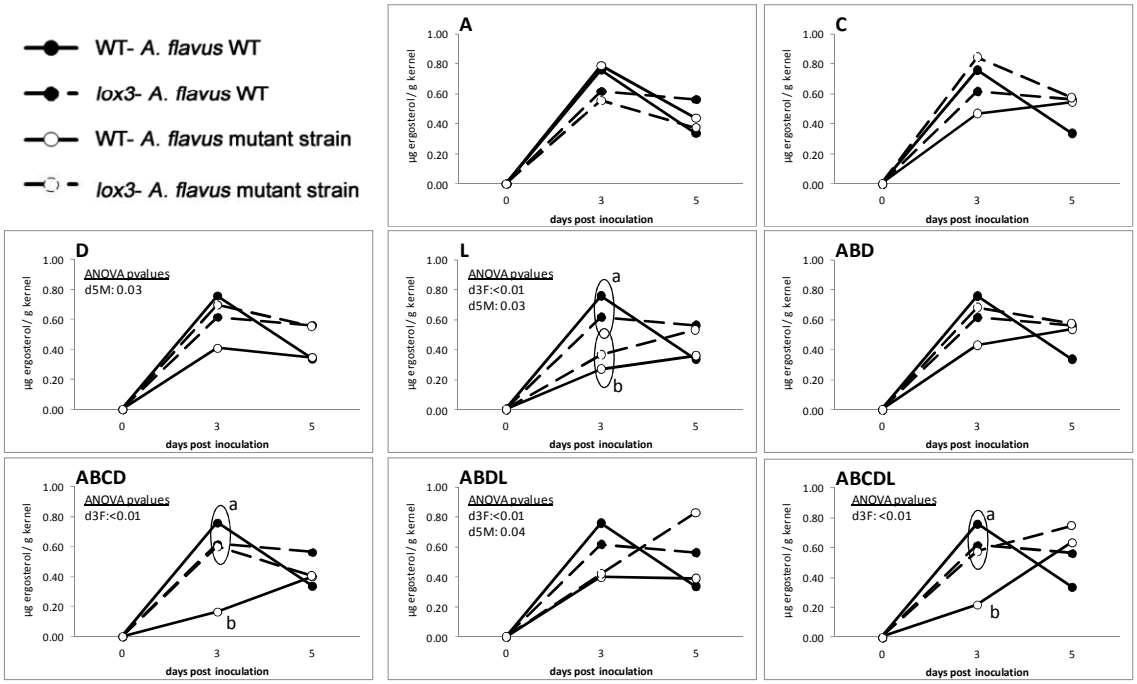


Fig. 12. Colonization of maize WT and *lox3* mutant kernels by *Aspergillus flavus* WT and oxylipin-deficient mutant strains. The Y-axis represents ergosterol content (µg / g kernel) and X-axis represents days post inoculation (dpi). Each panel is labeled with respective *A. flavus* mutant strain under consideration. Solid lines depict WT maize kernels, dashed lines depict *lox3* mutant kernels, black points represent means of WT *A. flavus*, and white points represent *A. flavus* mutant strains. Unconnected letters next to means indicate statistically significant differences ($p < 0.05$) by Tukey's HSD test. Asterisks represent changes in means between day 3 and day 5 ($p < 0.013$); statistically significant main effects are inserted when appropriate.

Aflatoxin

For all the strains and maize genotypes, AF was significantly increased from 3 to 5 dpi. Unlike time-dependent differences in AF levels, surprisingly few statistical changes were observed for biomass-dependent AF production for different fungal single and multiple gene mutants, with the notable exception of A and ABDL strains (Fig. 12). The lack of a functional *LOX3*-dependent process appeared to increase AF production in ABDL. Strain A had significantly reduced AF compared to WT *A. flavus* at 3 and 5 dpi. Despite the perturbation of AF production of strain A, it maintained a strong negative correlation between colonization and AF production (rho = -0.84, p = 0.03) comparable to those of WT (rho = -0.82, p = 0.02) (Table 2). In contrast, this correlation was lost for all other strains except C.

Pearson's correlation analysis was employed on the entire dataset to uncover relationships between these fungal processes. A negative correlation between colonization and conidiation (rho = -0.52, $p < 0.01$) at 3 dpi and a positive correlation between conidia and AF (rho = 0.34, $p < 0.01$) at 5 dpi were identified (Fig. 13). When correlations were assessed by fungal genotype, strains A and ABCD maintained a negative relationship between conidiation and colonization at day 3 (respectively, rho = -0.84, -0.99, $p < 0.01$, <0.01; Table 2). Strain A and ABCDL maintained positive relationship between conidiation and AF accumulation at 3 and 5 dpi, respectively (respectively, rho = 0.82, 0.76, p = 0.02, 0.02; Table 2). A negative correlation between

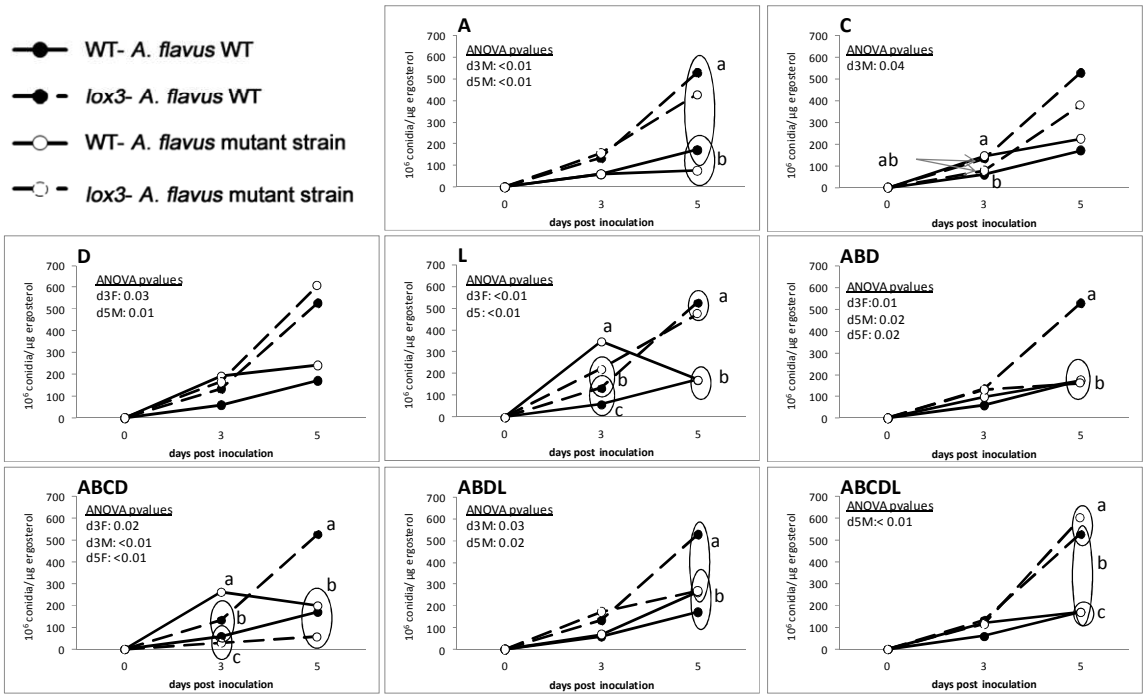


Fig. 13. Conidiation of *Aspergillus flavus* WT and oxylipin-deficient mutant strains on maize WT and *lox3* mutant kernels. The Y-axis represents conidiation (10^6 conidia/ µg ergosterol) and the X-axis represents days post inoculation (dpi). Each panel is labeled with the respective mutant *A. flavus* strain under consideration. Solid lines depict WT maize kernels, dashed lines depict *lox3* mutant kernels, black points represent means of WT *A. flavus*, and white points represent means of mutant strain. Unconnected letters next to means indicate statistically significant differences ($p < 0.05$) by Tukey's HSD test. Asterisks represent changes in means between day 3 and day 5 ($p < 0.01$); statistically significant main effects are inserted when appropriate.

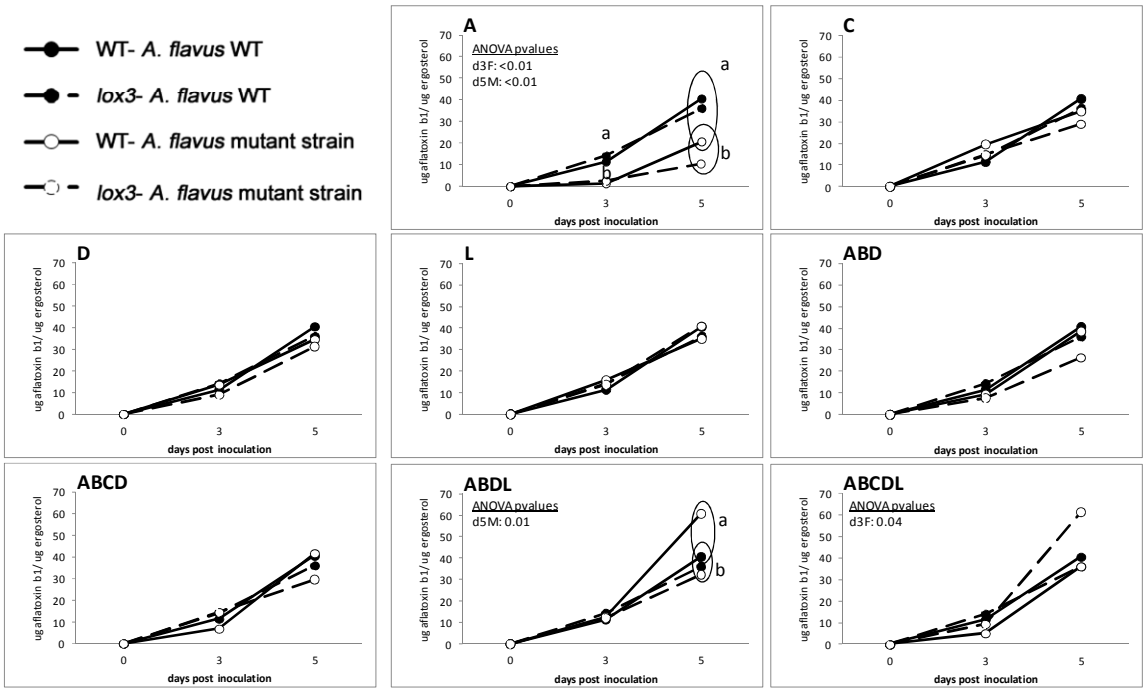


Fig. 14. AF content in maize WT and *lox3* mutant kernels upon infection with *Aspergillus flavus* WT and oxylipin-deficient mutant strains in kernel bioassay. The Y-axis represents content of AF (μg/ μg ergosterol) and X-axis represents days post inoculation (dpi). Each panel is labeled with the respective mutant *A. flavus* strain under consideration. Solid lines depict WT maize kernels, dashed lines depict *lox3* mutant kernels, black points represent means of WT *A. flavus*, and white points represent means of mutant strain. Unconnected letters next to means indicate statistically significantly differences ($p < 0.05$) by Tukey's HSD test. Asterisks represent changes in means between day 3 and day 5 ($p < 0.013$); statistically significant main effects are inserted when appropriate.

Grouping criteria		dpi	Pearson correlation				
Grouping by maize	by fungi		X	Y	Rho	p-value	# of samples
All samples	All	D3	colonization	conidia	-0.5204	0.0000	60
WT	All	D3	colonization	conidia	-0.6225	0.0002	31
All	ABCD	D3	colonization	conidia	-0.9853	0.0003	6
All	All	D5	conidia	aflatoxin	0.3387	0.0054	66
WT	All	D5	conidia	aflatoxin	0.4833	0.0059	31
lox3	All	D3	colonization	conidia	-0.4975	0.0060	29
All	A	D3	colonization	conidia	-0.8400	0.0090	8
All	A	D3	colonization	aflatoxin	-0.8754	0.0098	7
lox3	All	D5	conidia	aflatoxin	0.3838	0.0228	35
All	A	D3	conidia	aflatoxin	0.8210	0.0236	7
All	WT	D5	colonization	aflatoxin	-0.8206	0.0237	7
All	ABCDL	D5	conidia	aflatoxin	0.7600	0.0286	8
All	A	D5	colonization	aflatoxin	-0.8466	0.0335	6
All	C	D5	colonization	aflatoxin	-0.7713	0.0423	7

Table 3. Spearman's correlation of colonization, conidiation, and aflatoxin accumulation in WT and *lox3* mutant kernels infected by *A. flavus* WT and oxygenase mutant strains.

colonization and AF accumulation was seen in *A. flavus* WT and strains A and C (Table 2).

Phytohormone alterations in response to fungal infection

To understand the role of host and pathogen oxylipin signaling in the regulation of plant hormone biosynthesis during the *Aspergillus* -maize interaction, we simultaneously quantified abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA), and jasmonate-isoleucine conjugate (JA-Ile) in WT and *lox3* mutant kernels infected with WT and all oxylipin-deficient strains of *A. flavus* through LC-MS/MS.

The effects of maize genotype, fungal genotype, dpi within maize genotype, and maize x fungus genotypes on phytohormones were examined via ANOVA. Overall, maize genotype significantly affected IAA content, fungal genotype significantly affected IAA and JA-Ile levels, and the maize by fungal genotype combination significantly affected ABA and JA. In addition, all hormone levels were affected significantly by time after inoculation.

Among all the hormones measured, ABA was the most strongly affected by the host and fungal genotypes (Fig. 14). ABA content increased in WT kernels at 3 dpi and these levels maintained to at least 5 dpi; however, in *lox3* mutant kernels, there was an initial decrease in ABA content observed at 3 dpi but it rapidly recovered to reach levels comparable to WT by 5 dpi. While displaying a normal response at 3 dpi, strains A and

ABCD were unable to induce an ABA increase in *lox3* at 5 dpi. Strain L seemed to have an intermediate response at 5 dpi.

The dependency of ABA content on fungal and host genotype warranted correlation analysis to the previously collected fungal parameters. A strong and significant negative relationship was uncovered between ABA content and conidiation (rho = -0.75, p = 0.02), however, no significant correlations between ABA and colonization or AF biosynthesis were observed.

Unexpectedly, lox3 mutants produced greater levels of IAA compared to WT kernels, regardless of fungal genotype (Fig. 15).

Lox3 gene expression and its correlation with fungal parameters

To examine the effect of fungal-derived oxylipins on host metabolism, we analyzed the expression of *LOX3* in WT kernels infected by WT and oxylipin-deficient strains of *A. flavus*. Quantitative real-time PCR analysis of *LOX3* expression in WT kernels 3 dpi showed a general suppression of expression when kernels were inoculated with single mutations strains of *A. flavus,* compared to WT or mock-treated (Fig. 16A). When infected by ABD and ABCD, WT kernels displayed an increased expression of *LOX3* compared with WT *A. flavus* infected kernels. Incidentally, the expression of *LOX3* was negatively correlated with colonization (Fig. 16B).

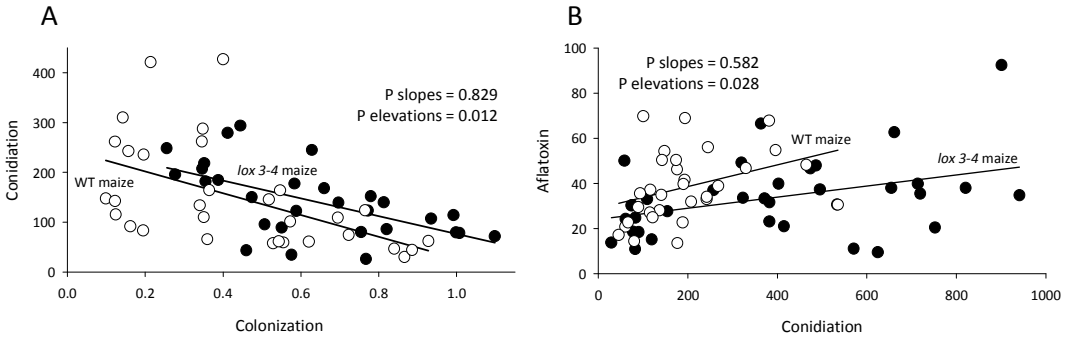


Fig. 15. Correlation of fungal parameters. (A) At 3 dpi, fungal conidiation decreased with increasing colonization in nine fungal genotypes (see Text) growing on either WT (empty circles) or *lox3* mutant (filled circles). Conidiation was significantly greater at similar colonization on *lox3* mutant kernels compared to WT (P = 0.012), while the rates of conidiation decrease with colonization were similar on both maize genotypes (p = 0.829) (*lox3* mutant maize: $y = 4.40 – 0.76x$, $r^2 = 0.258$, p = 0.005; WT maize: $y = 4.19 – 0.58x$, $r^2 = 0.333$, $p < 0.001$) (B) At 5 dpi, fungal AF production increased with increasing conidiation in nine fungal genotypes (see Text) growing on either WT kernels (empty circles) or *lox3* mutant (filled circles). AF production was significantly lower at similar conidiation on *lox3* mutant maize compared to WT maize (p = 0.028), while the rates of AF increase with conidiation were similar on both maize genotypes (p = 0.582) (*lox3 mutant* maize: $y = 2.50 + 0.56x$, $r^2 = 0.147$, P = 0.023; wild-type maize: $y = 0.79 + 1.03x$, $r^2 = 0.234$, $p < 0.006$) (actual data are shown; statistical analyses conducted on ln-transformed conidiation and √-transformed AF values)

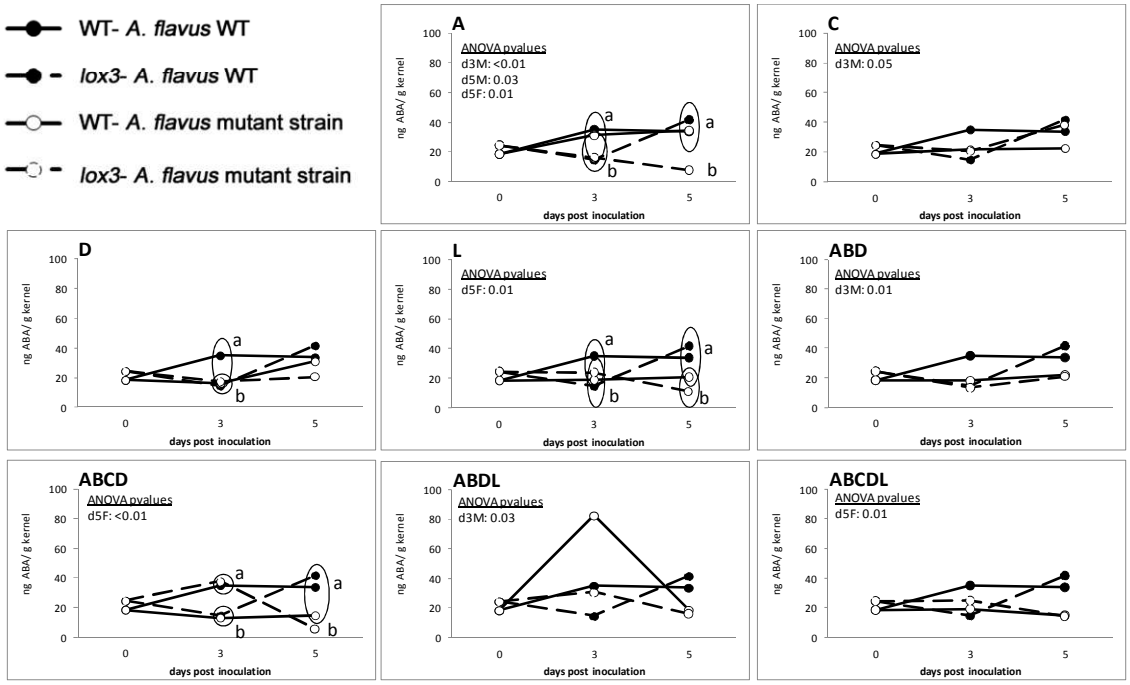


Fig. 16. Abscisic acid (ABA) content in maize WT and *lox3* mutant kernels upon infection with *Aspergillus flavus* WT and oxylipin-deficient mutant strains. The Y-axis represents ABA content (ng/ g kernel) and X-axis represents days post inoculation (dpi). Each panel is labeled with respective *A. flavus* mutant strain under consideration. Solid lines depict WT maize kernels, dashed lines depict *lox3* mutant kernels, black points represent means of WT *A. flavus*, and white points represent means of mutant strain. Unconnected letters next to means indicate statistically significant differences ($p < 0.05$) by Tukey's HSD test. Asterisks represent changes in means between day 3 and day 5 ($p < 0.013$); statistically significant main effects are inserted when appropriate.

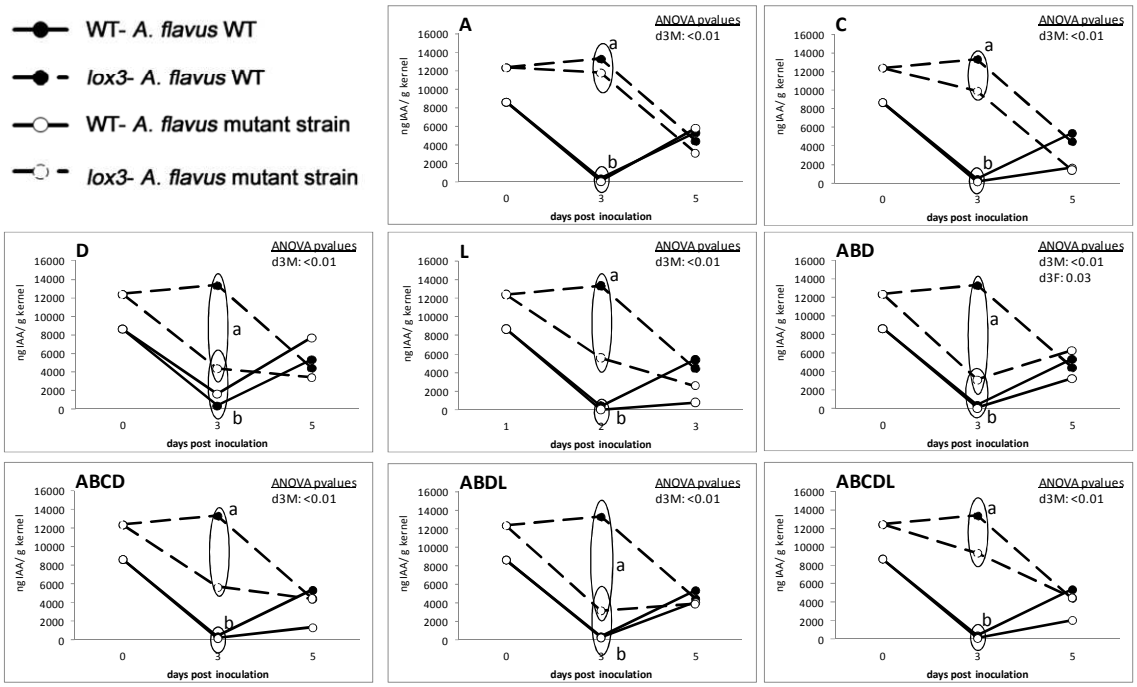


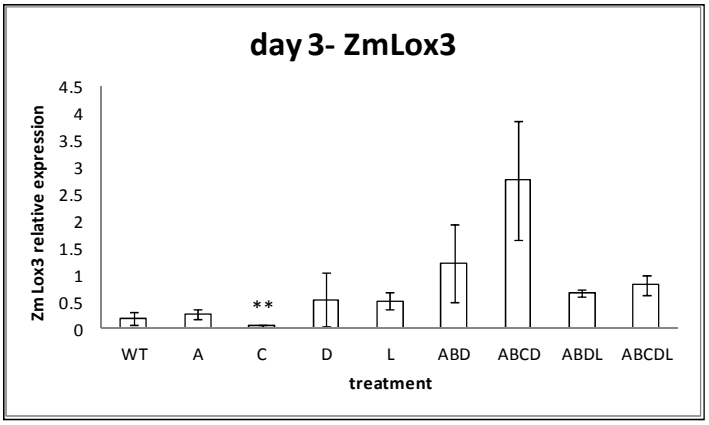
Fig. 17. Indole-3-acetic acid (IAA) content in maize WT and *lox3* mutant kernels upon infection with WT and *Aspergillus flavus* oxylipin-deficient mutant strains. The Y-axis represents IAA content (ng/ g kernel) and X-axis represents days post inoculation (dpi). Each panel is labeled with respective mutant *A. flavus* strain under consideration. Solid lines depict WT maize kernels, dashed lines depict *lox3* mutant kernels, black points represent means of WT *A. flavus*, and white points represent means of mutant strain. Unconnected letters next to means indicate statistically significant differences ($p < 0.05$) by Tukey's HSD test. Asterisks represent changes in means between day 3 and day 5 ($p < 0.02$); statistically significant main effects are inserted when appropriate.

Hormone ratio correlation with fungal parameters

Cross-talk and complex interactions among phytohormones are well established phenomena (Robert-Seilaniantz et al., 2011); the relative hormone signature, as the individual hormones themselves, is quickly gaining appreciation for its role in regulating diverse physiological processes (Pirasteh-Anosheh et al., 2013). We examined ratios between all hormones analyzed and correlated them to the examined fungal parameters (Table 3). Remarkably, we identified strong correlation between specific hormone ratios and fungal pathogenicity phenotypes. ABA/ JA was slightly negatively correlated with colonization (rho = -0.33, p-value= 0.05). Both ABA/JA-Ile (rho = 0.44 p = < 0.01) and IAA/JA-Ile (rho = 0.41 p = 0.01) were positively correlated with conidiation.

ABA/ JA, ABA/JA-Ile, IAA/ JA, and IAA/JA-Ile were all positively and strongly correlated with AF accumulation (respectively, rho = 0.46, 0.80, 0.62, 0.75, p<0.01, <0.01, <0.01, <0.01) (Table 4). Surprisingly, there is a correlation of JA/ JA-Ile to conidiation and AF (rho = 0.49, -0.584, p < 0.01, <0.01), suggesting that additional jasmonates (e.g., methyl-JA) may contribute towards these fungal processes during infection of seed. These observations suggest a complex ABA and jasmonate interaction, which may govern AF regulation. These observations warrant further exploration and may provide maize breeders and industry biochemical markers for reducing AF contamination.

A



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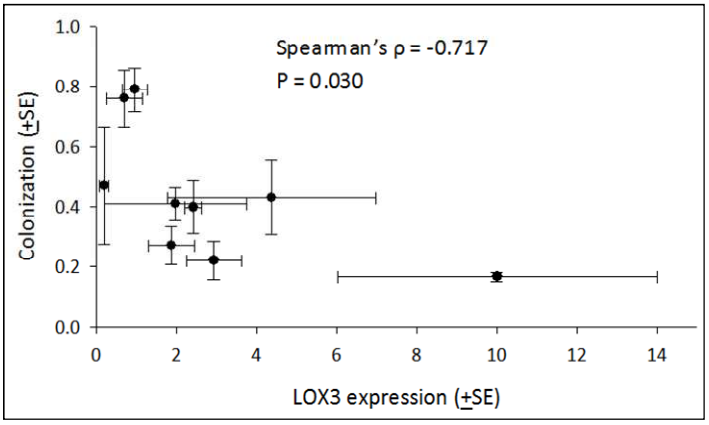

Fig. 18. Role of *LOX3* in the interaction of fungus oxylipin mutants. (A) Expression pattern of *ZmLox3* in response to various *Aspergillus flavus* oxylipin mutant strains. The 2-ΔΔCT method was employed to measure relative gene expression, with expression of the mock samples inoculated used as controls. Bars represent expression means of three biological replicate samples ± SE. Dunnett's test on ranks was used to compare expression against WT ($p < 0.01$). (B) Correlation of *ZmLox3* expression and colonization of WT and mutant *A. flavus* strains on WT maize kernel. Each point represents respective *A. flavus* strains' *ZmLox3* expression means against colonization ± SE. Spearman's correlation test (rho = -0.717, p = 0.03).

DISCUSSION

Taken together, this study suggests that the specific combination of plant and fungal genotypes and the resulting oxylipin bouquet affect disease outcomes. Fungal LOX-derived oxylipins were revealed important for normal colonization of maize kernels and fungal LOX-mediated colonization is in part dependent on suppression of host LOX3-derived defense signaling. In terms of conidiation, functional PpoD appears to be a strong negative regulator of conidiation. The conidiation stagnation of ABD and ABCD by 5 dpi, suggests these functional enzymes are required for *LOX3*-dependent conidiation induction. Furthermore, the partial restoration of ABD and ABCD through *loxA* deletion suggests antagonism between *ppos* and *loxA* during regulation of conidiation. This analysis uncovered previously unknown specialized role of *ppoA* as a positive regulator of AF biosynthesis. Unlike a previous report which described *ΔppoA* mutants producing increased levels of AF relative to WT (Brown et al., 2009), our observations match closely with earlier reports of *ΔppoA* mutants from *A. nidulans* in the regulation of sterigmatocystin, the penultimate precursor to AF (Tsitsigiannis and Keller, 2006). This suggests *ppoA* has a conserved role in mycotoxin regulation in Aspergilli and the conflicting observations are likely due to the inoculation and incubation differences among the methods (e.g., volatile accumulation in humidity chambers). The finding that PpoA is uniquely involved in the regulation of AF biosynthesis is fundamental to understanding oxylipin fungal biology and mycotoxin production and warrants further studies involving additional maize inbred lines,

especially those that show contrasting levels of contamination with AF greenhouse/ field conditions. The correlation between the fungal parameters (Fig. 13) suggests that during ideal incubation conditions, *A. flavus* diverts its energy towards vegetative growth as opposed to stressed conditions, which drive energy towards conidiation. These observations provide, for the first time, statistical support for previous anecdotal suspensions of a balance between fungal vegetative growth and sporulation in *A. flavus*. This is an especially strong case given that both maize genotypes are near-isogenic and all fungal genotypes are isogenic. A negative correlation between colonization and AF accumulation was seen in *A. flavus* WT and strains A and C (Table 2). This suggests a role for plant and fungal oxylipins in regulating and maintaining the normal balance between colonization, conidiation, and AF production and warrants further molecular and biochemical exploration of the fungal strains in which this balance is not maintained any longer.

Variable	by Variable	Spearman ρ	Prob>|ρ|
colonization mean	ABA/JA	-0.3312	0.0485
conidiation mean	ABA/JA-Ile	0.4388	0.0074
conidiation mean	IAA/JA-Ile	0.4116	0.0126
conidiation mean	JA/JA-Ile	0.4904	0.0028
aflatoxin	ABA/JA-Ile	0.7985	<.0001
aflatoxin	IAA/JA	0.6214	<.0001
aflatoxin	IAA/JA-Ile	0.7523	<.0001
aflatoxin	ABA/JA	0.4638	0.0044
aflatoxin	JA/ABA	-0.584	0.0002
aflatoxin	JA/JA-Ile	0.7341	<.0001

Table 4. Spearman's correlation of hormone ratios with fungal parameters of WT and *lox3* mutant kernels infected by *A. flavus* WT and oxygenase mutant strains.

One of the unexpected and important findings of this study is that, overall, *lox3* mutants produced greater levels of IAA compared to WT kernels, regardless of fungal genotype (Fig. 15). These results, to the best of our knowledge, show for the first time that a LOX isoform that preferentially inserts oxygen at carbon position 9 rather than 13 (as required for JA synthesis), may negatively regulate IAA biosynthesis, at least in seed. Further analysis of the main effect of maize genotype showed that *lox3* mutants accumulated increased levels of IAA compared to WT kernels, with levels most prominent at 3 dpi. The observation that *lox3* mutant kernels are deficient in several oxylipins (e.g., 9-HOD/TE and 9-KOD/TE) (Gao et al., 2008b), suggests that one, several, or all of these oxylipins may directly or indirectly negatively regulate auxin biosynthesis. Auxin has been recognized for its role in modulating plant pathogen interactions with most published studies showing that auxin facilitate disease progression (Kidd et al., 2011; Mutka et al., 2013). Therefore, it is possible that increased IAA levels in the *lox3* mutant may be one potential mechanism behind increased susceptibility of the mutant to *A. flavus* (this study and Gao et al. 2009) and *A. nidulans* (Gao et al., 2009).

In terms of ABA production, this study suggests that functional PpoA, B, C and D are important for the normal ABA response of maize kernels to *Aspergillus* infection. Despite displaying a normal response at 3 dpi, strains A and ABCD were unable to induce ABA increase in *lox3* at 5 dpi. However, no significant correlations between ABA and colonization or AF biosynthesis were observed, suggesting a specific effect of ABA upon conidiation. Despite numerical differences, no significant differences were

observed in ABA content of strains C, ABD, ABDL, and ABCDL, implicating the respective intact fungal genes as essential for normal regulation of ABA upon *A. flavus* infection. The notion that *Aspergillus* produces ABA independently of maize, as was seen with *Botrytis cinerea* and *Ustilago maydis* (Siewers et al., 2004; Bruce et al., 2011), cannot be discounted

When infected by ABD and ABCD, WT kernels displayed an increased expression of *LOX3*, suggesting that in combination, functional fungal *ppo* genes are required to suppress *LOX3* during infection. The additive effect of multiple Ppos in regulation of host lipid metabolism was previously documented in studies with *A. nidulans* and peanut, where multiple Ppos were required to substantially decrease host expression of a 13-LOX (Brodhagen et al., 2008). Additionally, in this study, fungal colonization was negatively correlated with the expression of maize *LOX3* (Fig. 16B), suggesting that the ability of *A. flavus* to successfully colonize kernels depends on its ability to suppress *LOX3* expression through multiple Ppo genes. This observation would be expected of a defense gene to be regulated in this manner in a susceptible line such as B73, the genetic background of the WT and *lox3* mutant.

Hormone ratio correlation with fungal parameters

Cross-talk and complex interactions among phytohormones are well established phenomena (Robert-Seilaniantz et al., 2011); the relative hormone signature, as the

individual hormones themselves, is quickly gaining appreciation for its role in regulating diverse physiological processes (Pirasteh-Anosheh et al., 2013). We examined ratios between all hormones analyzed and correlated them to the examined fungal parameters (Table 2). Remarkably, we identified strong correlation between specific hormone ratios and fungal pathogenicity phenotypes. ABA/ JA was slightly negatively correlated with colonization (rho = -0.33, p-value= 0.05). Both ABA/JA-Ile and IAA/JA-Ile were positively correlated with conidiation (respectively, rho = 0.44, 0.41, $p < 0.01$, 0.01). ABA/ JA, ABA/JA-Ile, IAA/ JA, and IAA/JA-Ile were all positively and strongly correlated with AF accumulation (respectively, rho = 0.46, 0.80, 0.62, 0.75, $p < 0.01$, <0.01, <0.01, <0.01). Surprisingly, there is a correlation of JA/ JA-Ile to conidiation and AF (rho = 0.49, -0.584, $p < 0.01$, <0.01), suggesting that additional jasmonates (e.g., methyl-JA) (Gfeller et al., 2010) may contribute towards these fungal processes during infection of seed. These observations suggest a complex ABA and jasmonate interaction, which may govern AF regulation.

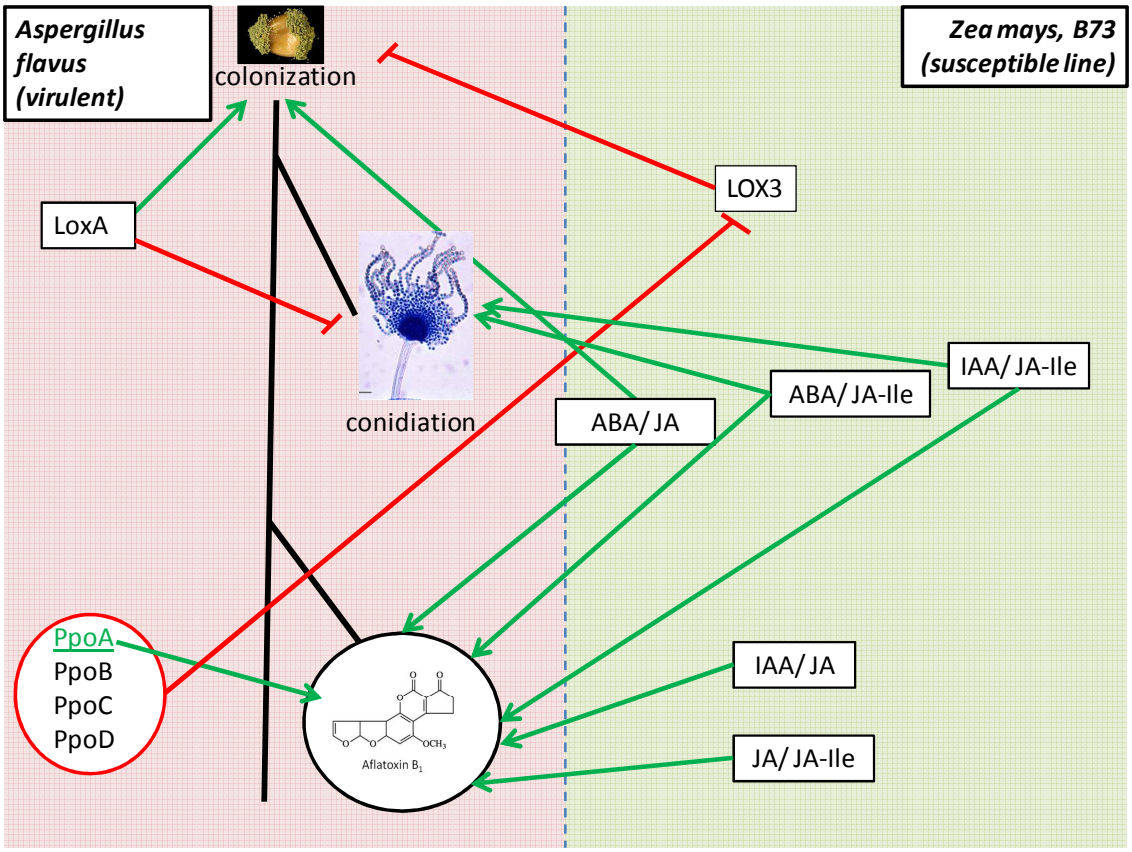

Fig. 19. Interaction between LOX3 and *A. flavus* fungal oxygenases (LoxA and Ppo genes) during colonization, conidiation, and aflatoxin accumulation of maize seed. The sole fungal LOX positively regulates colonization and negatively regulates conidiation. PpoA positively regulates aflatoxin accumulation. Multiple Ppo genes function together to suppress LOX3 gene expression, which in turn suppresses colonization. The hormone ratios of ABA/ jasmonates and IAA/ jasmonates positively correlate with aflatoxin accumulation. Other ratios also correlated positively with fungal process: ABA/JA- colonization; ABA/ JA-Ile- conidiation; IAA/JA-Ile- conidiation. JA/JA-Ile also correlated positively with aflatoxin accumulation. Green lines indicate positive regulation and red lines indicate negative regulation.

CHAPTER V

ROLE OF MAIZE LOX7 AND *ASPERGILLUS FLAVUS* OXYLIPIN BIOSYNTHESIS GENES IN SEED-PATHOGEN INTERACTIONS

INTRODUCTION

Lipoxygenases (LOX), non-heme iron-containing dioxygenases are the primary producers of a group of oxygenated fatty acids, which function as potent inter- and intra-cellular signals in plants (Feussner and Wasternack, 2002). These molecules, known as oxylipins regulate diverse processes related to development and defense against biotic and abiotic stress. Previously, it was shown that maize LOX3 was required for defense against *Aspergillus* seed rot (Gao et al., 2009) and initiate a general defense mechanisms against seed colonization (see Chapter III and IV). In the maize B73 inbred line, LOX3 is part of a member of a thirteen LOX genes in maize genome, classified largely into two groups, depending on positional specificity of oxygen incorporation into fatty acid carbon chains.

In our mutant screen for maize genes involved in the regulation of the mycotoxin production (Chapter III), there was only one other LOX gene, *LOX7*, which appeared to be involved in defense against this pathogen. Unlike *LOX3* which is a 9-LOX (Gao et al., 2008b), *LOX7* belongs to a subfamily of 13-LOXs (Christensen et al., 2013). LOX7 isoform is the closest paralogue to LOX8 (also known as tasselseed1) the LOX

responsible for the production of JA in tassel primordia during sex determination (Acosta et al., 2009). However, the function of LOX7 in maize remains to be elucidated.

Since *lox7* mutants are more susceptible to colonization at both 3 and 5 dpi, we decided to explore the potential involvement of *LOX7* in lipid-mediated signaling with *A. flavus*. For this, maize WT and *lox7* mutants were infected by WT *A. flavus* and a collection of diverse oxylipin-deficient mutant strains: *ΔppoA*, *ΔppoC*, *ΔppoD*, and *Δlox*. Following the format of Chapter IV, single letters designated these mutants (Table 1). I have tested the involvement of each of the fungal genes and maize *LOX7* gene in regulation of the fungal processes of colonization, conidiation, and AF accumulation. To understand the molecular and biochemical pathways responsible for altered phenotypes of these mutants we measured content of several phytohormones including ABA, IAA, JA, and JA-Ile.

RESULTS

Effect of host and fungal mutations on colonization

Ergosterol was used as an accurate representation of fungal biomass because this lipid is a major component of fungal cell membranes and is not present in plant cells. In terms of colonization, at 3 dpi, *lox7* mutants had a 3.9-fold increase in the biomass of the

fungus, regardless of the strain (Fig. 20), suggesting that *LOX7* is required for general defense against this pathogen.

Increased susceptibility of *lox7* mutants was also evident at day 5 for WT, A, C, and L fungal strains. Strain D that did not show a statistically significant in colonization between WT and *lox7* mutants at 5 dpi.

Effect of host and fungal mutations on conidiation

In terms of conidiation, *lox7* mutants supported a precocious increase in the number of conidia per a unit of biomass at 3 dpi, compared with WT kernels but not at 5 dpi. This suggests that functional LOX7 is responsible for normal inhibiting fungal conidiation at early stages of disease development.

In terms of the effect of fungal oxylipin mutants, statistical analysis of the entire set of data suggested that fungal genotypes significantly affected conidiation at both 3 and 5 dpi ($p < 0.0001$ and $p = 0.0072$, respectively). Strain A consistently produced less conidia at both 3 and 5 dpi. Compared with WT kernels, conidiation of strain A was increased when grown on *lox7* mutant kernels at 3 dpi, but remained the same at 5 dpi (Fig. 21), suggesting precocious conidiation of strain A on *lox7* mutants.

Effect of host and fungal mutations on aflatoxin accumulation

Aflatoxin accumulation followed a pattern similar to conidiation ($p = 0.0029$, $p < 0.0001$, respectively 3 and 5 dpi). Similar to conidiation, kernels infected by strain A accumulated less aflatoxin at 3 and 5 dpi compared with kernels infected by WT strain (Fig. 22). This suggests that PpoA is required for normal aflatoxin accumulations in both genetic backgrounds. Overall, LOX7 does not appear to play a major role in the regulation of aflatoxin production, reminiscent to LOX3 results (see Chapter IV).

Effect of oxylipin mutations on content of plant hormones

To understand the role of LOX7 in the regulation of phytohormones during *A. flavus* infection of seed, the following hormones were measured, ABA, IAA, JA, and JA-Ile. At 3 dpi, only numerical differences were observed in ABA content for all fungal genotypes regardless of maize genotype but none were statistically significant (Fig. 23). The only statistical difference was observed for *lox7* mutants infected by strain A and C. Both strains induced greater levels of ABA on *lox7* mutants compared to WT at 5 dpi. When averaged between all the fungal and plant genotypes, *lox7* mutants produced more ABA in response to pathogen infection. This suggests LOX7 acts to suppress ABA a phenomenon more evident at later disease stages.

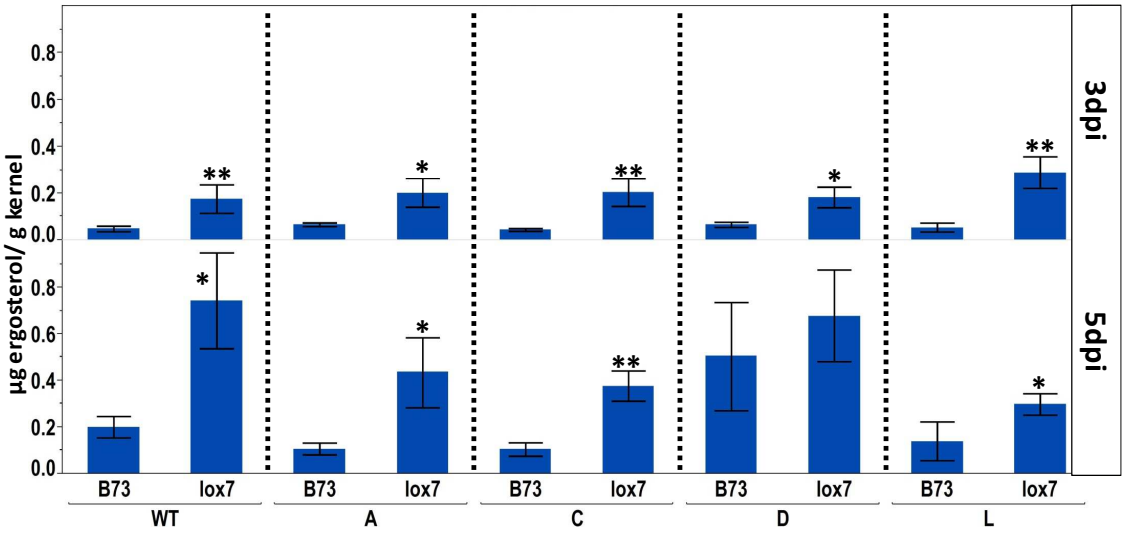

Fig. 20. Colonization of maize WT and lox7 mutant kernels by *Aspergillus flavus* WT and oxylipin-deficient mutant strains. The Y-axis represents ergosterol content (µg / g kernel) and the X-axis represents maize (B73 vs *lox7*) verses fungal (WT, A, C, D, L) genotype. The top and the bottom halves of the graph represent 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant differences between WT and *lox7* mutant kernels for each fungal strain analyzed within the same time-point (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

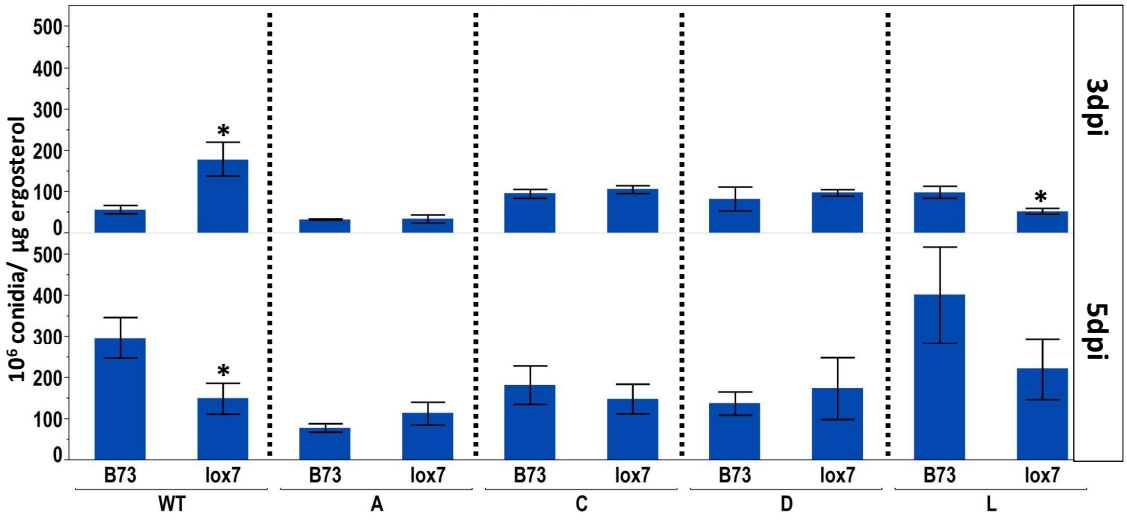

Fig. 21. Conidiation of *Aspergillus flavus* WT and oxylipin-deficient mutant strains on WT and *lox7* mutant maize kernels. The Y-axis represents fungal-biomass dependent conidiation (10^6 spores/ µg ergosterol) and the X-axis represents maize (B73 vs *lox7*) verses fungal (WT, A, C, D, L) genotype. The top and the bottom halves of the graph represent 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant differences between WT and *lox7* mutant kernels for each fungal strain analyzed within the same time-point (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

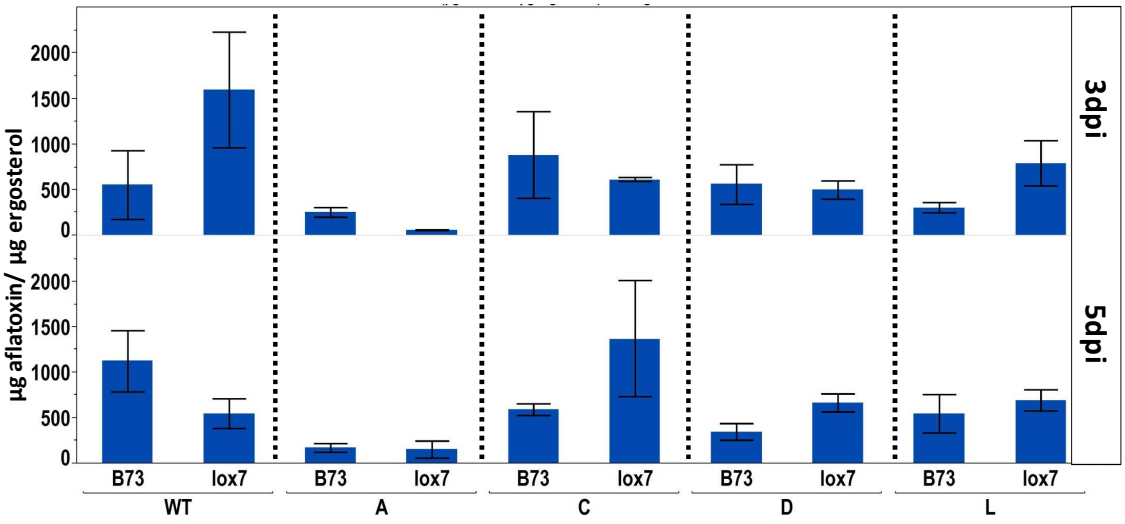

Fig. 22. Aflatoxin B_1 accumulation of maize WT and lox7 mutant kernels infected by *Aspergillus flavus* WT and oxylipin-deficient mutant strains. The Y-axis represents fungal-biomass dependent conidiation (10^6 spores/ µg ergosterol) and the X-axis represents maize (B73 vs *lox7*) verses fungal (WT, A, C, D, L) genotype. The top and the bottom halves of the graph represent 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant differences between WT and *lox7* mutant kernels for each fungal strain analyzed within the same time-point (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

Mock-control of *lox7* mutants accumulated significantly decreased levels of IAA compared with mock-treated WT kernels, suggesting LOX7 is required to suppress IAA accumulation during germination. When infected, *lox7* mutants contained decreased IAA levels at 3 dpi compared with WT (main effect $p = 0.0024$) (Fig. 24), suggesting LOX7 negatively modulates IAA accumulation during seed infection by *A. flavus*. Interestingly, *lox3* mutants, which are also susceptible to A. flavus, contained more IAA in infected seed. This indicates that IAA is not a major player in susceptibility to colonization by itself. This also prompted me to investigate whether ratios between different hormones could explain disease progression (Table 5). Similar to 3 dpi, fungal genotype had a main effect on IAA content at 5 dpi ($p = 0.0001$).

In terms of jasmonates, while no statistical difference was detected for each pairs compared between WT and *lox7*, when averaged across fungal strains, *lox7* kernels accumulated lower levels of both JA (main effect $p = 0.0014$) and JA-Ile (main effect $p < 0.0001$) compared with WT kernels at 3 dpi. This indicates that LOX7 is required for normal JA production, as predicted for a 13-LOX, especially during early stages of disease development. Interestingly, at 5 dpi, infection with strain L resulted in increased JA-Ile content compared with mock ($p = 0.0139$) (Fig. 25, 26), suggesting fungal LOX-products negatively regulate LOX7-mediated JA production.

Phytohormone correlations

Since no strong association of ABA, IAA, or JA was observed with colonization, conidiation, or aflatoxin accumulation, I hypothesized that neither of the hormones individually control these three processes, thus prompting the idea that ratios between different hormones maybe responsible for the phenotypes observed (Table 5). To test this hypothesis, I have done Spearman's rank correlation coefficient (also known as Spearman's rho) on the entire data set of both individual hormones and hormone ratios and fungal process in diverse combinations (see Methods). Remarkably, several hormone ratios strongly correlated with all three fungal biological processes assessed in this study. ABA and IAA positively correlated with colonization (rho = 0.4496, 0.6617, p = 0.0275, 0.0004), yet colonization negatively correlated with the ratio of IAA/JA (rho = -0.5426, p = 0.0062). Conidiation positively correlated with JA-Ile (rho = 0.6774 p = 0.0003) but correlated negatively with IAA (rho = -0.573 p = 0.0034). Additionally, the ratios between IAA/JA (rho = 0.5261 p = 0.0083) and ABA/JA (rho = 0.4635, p = 0.0225) positively correlated with conidiation. Finally, aflatoxin correlated positively with the hormone ratio of ABA/JA (rho = 0.473 p = 0.0196). To the best of my knowledge, correlation between fungal pathogenicity processes and hormone ratios was reported before.

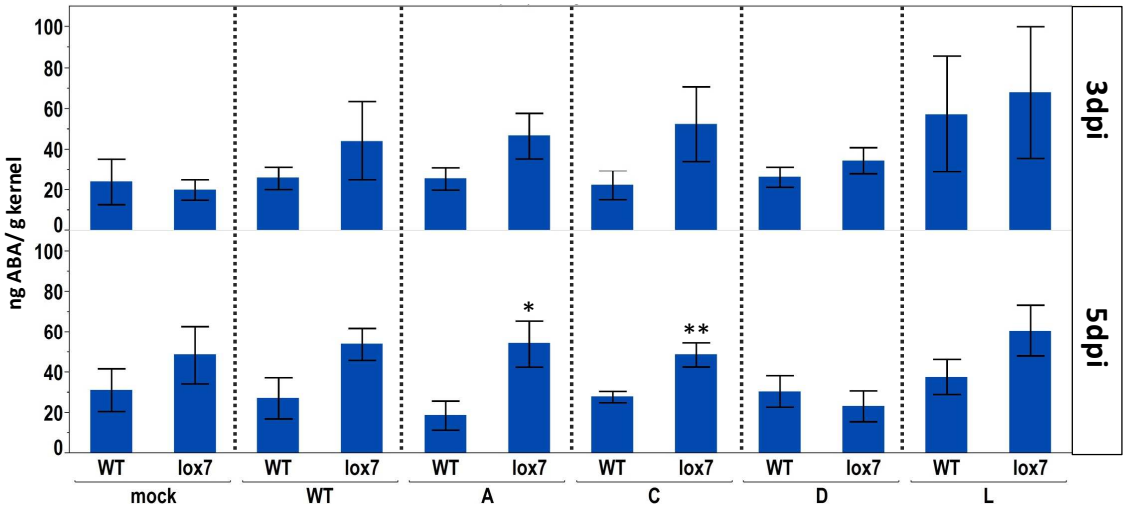

Fig. 23. ABA accumulation of *Aspergillus flavus* infected kernels of WT and *lox7* mutant kernels. The Y-axis represents phytohormone content per gram of kernel (ng phytohormone/ g kernel) and the X-axis represents maize (B73 vs *lox7*) verses fungal (mock, WT, A, C, D, or L) genotype. The top and the bottom halves of the graph represent 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant differences between WT and *lox7* mutant kernels for each fungal strain analyzed within the same time-point (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

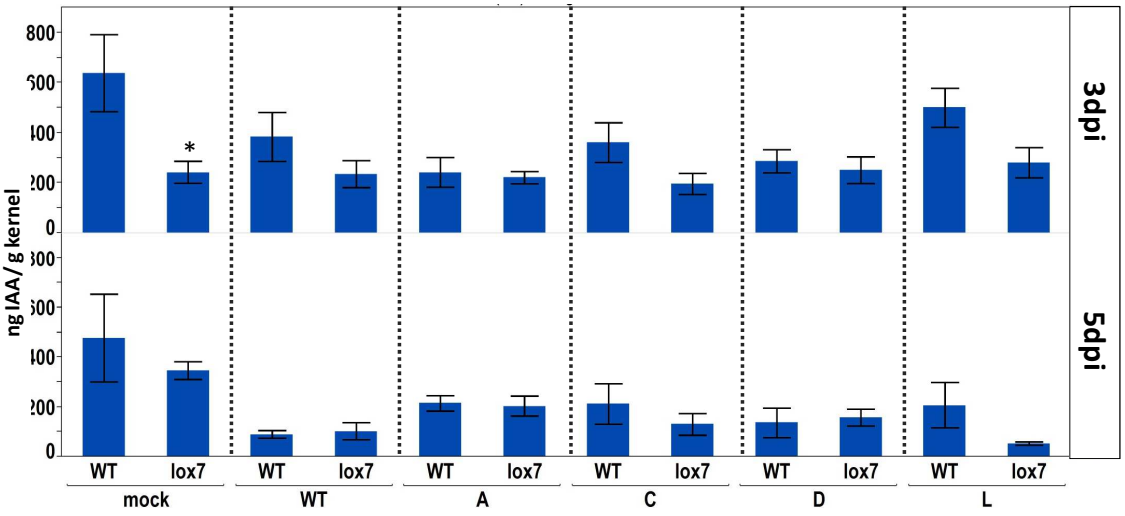

Fig. 24. IAA accumulation of *Aspergillus flavus* infected kernels of WT and *lox7* mutant kernels. The Y-axis represents phytohormone content per gram of kernel (ng phytohormone/ g kernel) and the X-axis represents maize (B73 vs *lox7*) verses fungal (mock, WT, A, C, D, or L) genotype. The top and the bottom halves of the graph represent 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant differences between WT and *lox7* mutant kernels for each fungal strain analyzed within the same time-point (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

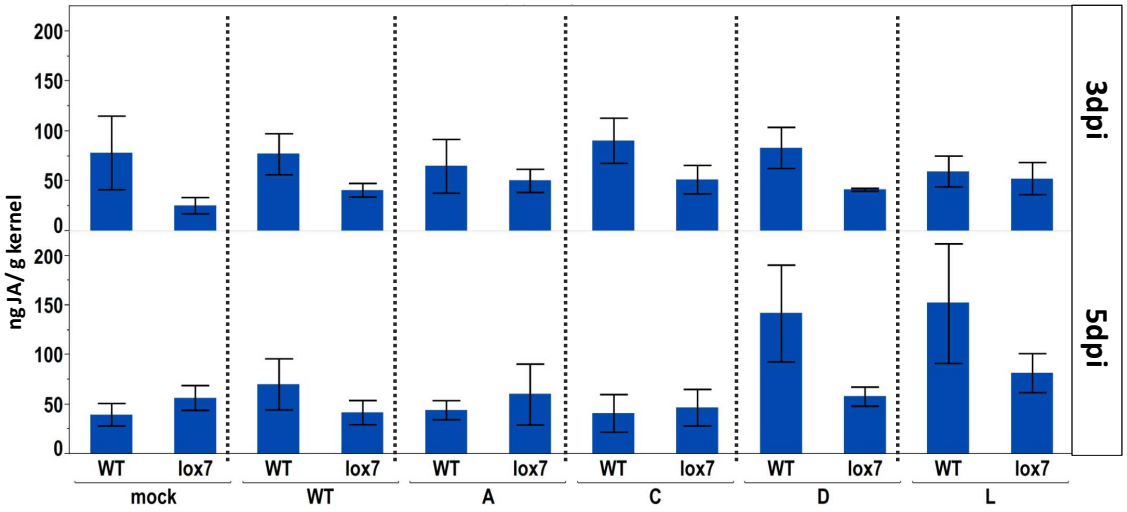

Fig. 25. JA accumulation of *Aspergillus flavus* infected kernels of WT and *lox7* mutant kernels. The Y-axis represents phytohormone content per gram of kernel (ng phytohormone/ g kernel) and the X-axis represents maize (B73 vs *lox7*) verses fungal (mock, WT, A, C, D, or L) genotype. The top and the bottom halves of the graph represent 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant differences between WT and *lox7* mutant kernels for each fungal strain analyzed within the same time-point (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

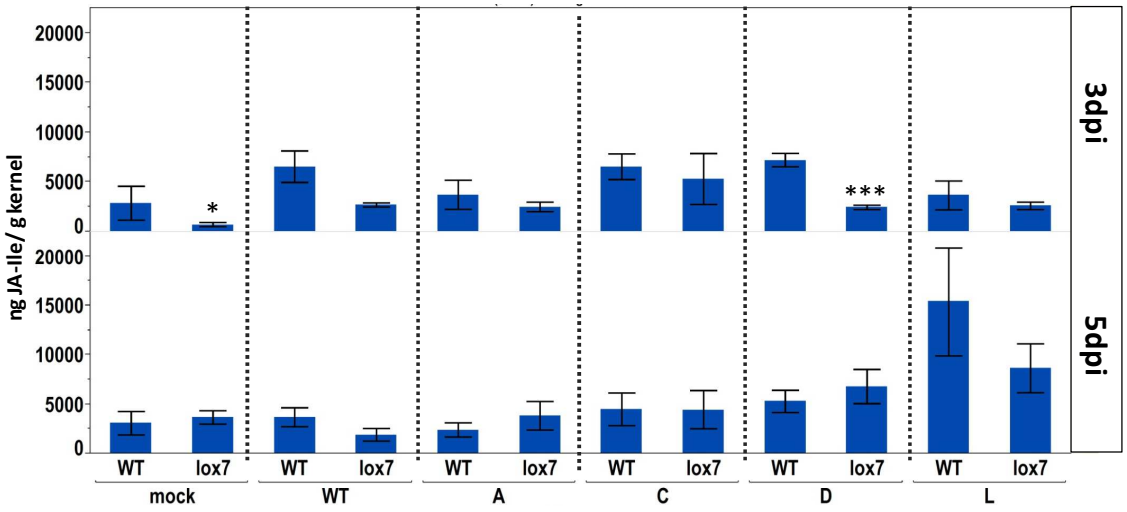

Fig. 26. JA-Ile accumulation of *Aspergillus flavus* infected kernels of WT and *lox7* mutant kernels. The Y-axis represents phytohormone content per gram of kernel (ng phytohormone/ g kernel) and the X-axis represents maize (B73 vs *lox7*) verses fungal (mock, WT, A, C, D, or L) genotype. The top and the bottom halves of the graph represent 3 and 5 days post inoculation (dpi), respectively. Bars are means ± SE; n=5. Asterisks represent statistically significant differences between WT and *lox7* mutant kernels for each fungal strain analyzed within the same time-point (Student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$)

Variable	by Variable	Spearman ρ	Prob>|ρ|
colonization	ABA	0.4496	0.0275
colonization	IAA	0.6617	0.0004
colonization	IAA/ JA	-0.5426	0.0062
conidiation	JA-Ile	0.6774	0.0003
conidiation	IAA	-0.573	0.0034
conidiation	IAA/ JA	0.5261	0.0083
conidiation	ABA/ JA	0.4635	0.0225
aflatoxin	ABA/ JA	0.473	0.0196

Table 5. Spearman's correlation of hormone ratios with fungal parameters of WT and *lox7* mutant kernels infected by *A. flavus* WT and oxygenase mutant strains.

DISCUSSION

To summarize these complicated results, I have generated a working model that visually explains the major findings in this Chapter (Fig. 27). One of the most important discoveries from this study is that LOX7 is required for defense against *A. flavus.* This is only the second report of any LOX required for defense against this pathogen. The other defensive LOX is LOX3 (see Chapter III and IV; and Gao et al. 2009). While LOX3 is predominantly a 9-LOX (Wilson et al., 2001), LOX7 is a 13-LOX (Christensen et al., 2013). This suggests that both types of regio-specific LOXs function in defense against *A. flavus.* Similar to LOX3, LOX7 suppresses colonization on nearly all strains of *A. flavus* tested, pointing to its role in a general defensive pathway that is largely independent fungal oxylipin pathways. One plausible explanation for similar role of two vastly different biochemical classes of LOXs is that the two genes may depend on each other transcriptionally or cross-talk down-stream of their primary products, which warrants further investigation. This speculation is based on our finding that the genes are show similar expression pattern in developing embryo (Fig. 10) with both of them expressed between 16 and 24 days after pollination. As shown in Figure 10, LOX7 appears to be expressed earlier and stronger than LOX3 at the same time after pollination, it is likely that LOX7-mediated JA may induced expression of LOX3. This notion has to be further investigated using single and double LOX mutants for both genes.

The role of LOX7 in conidiation appears to be in suppression of precocious sporulation. This conclusion is based on the observation that at early disease stages, *lox7* produced more conidia at 3 dpi, but less at 5 dpi.

Among the fungal lipoxygenase and Ppo mutants, strong evidence for the role in regulation of conidiation and aflatoxin accumulation was found only for PpoA. Once again, similarity can be drawn between LOX3 and LOX7 functions*:* In the LOX3 study (Chapter IV), PpoA appears to regulate aflatoxin biosynthesis at statistically significant level and conidiation, but only numerically (Fig. 27).

LOX7 appears to oppositely regulate ABA and IAA. Throughout the disease progression, LOX7 suppressed ABA accumulation (Fig. 23), but induced IAA especially during early disease stages (Fig. 24). Together, this suggests that LOX7 functions as a key regulatory point for cross-talk between these hormones in maize seed and hypothetically, by its direct involvement in JA biosynthesis. Interestingly, the ratios of ABA/ IAA and ABA/ JA are positively associated with colonization and aflatoxin accumulation, respectively. ABA is best known for its involvement in drought stress responses (Raghavendra et al., 2010). Because aflatoxin contamination of seed typically occurs in the driest years (Cotty and Jaime-Garcia, 2007), my finding that higher ABA is associated with greater aflatoxin may provide a mechanistic link drought and higher incidence of aflatoxin contamination of corn under field conditions.

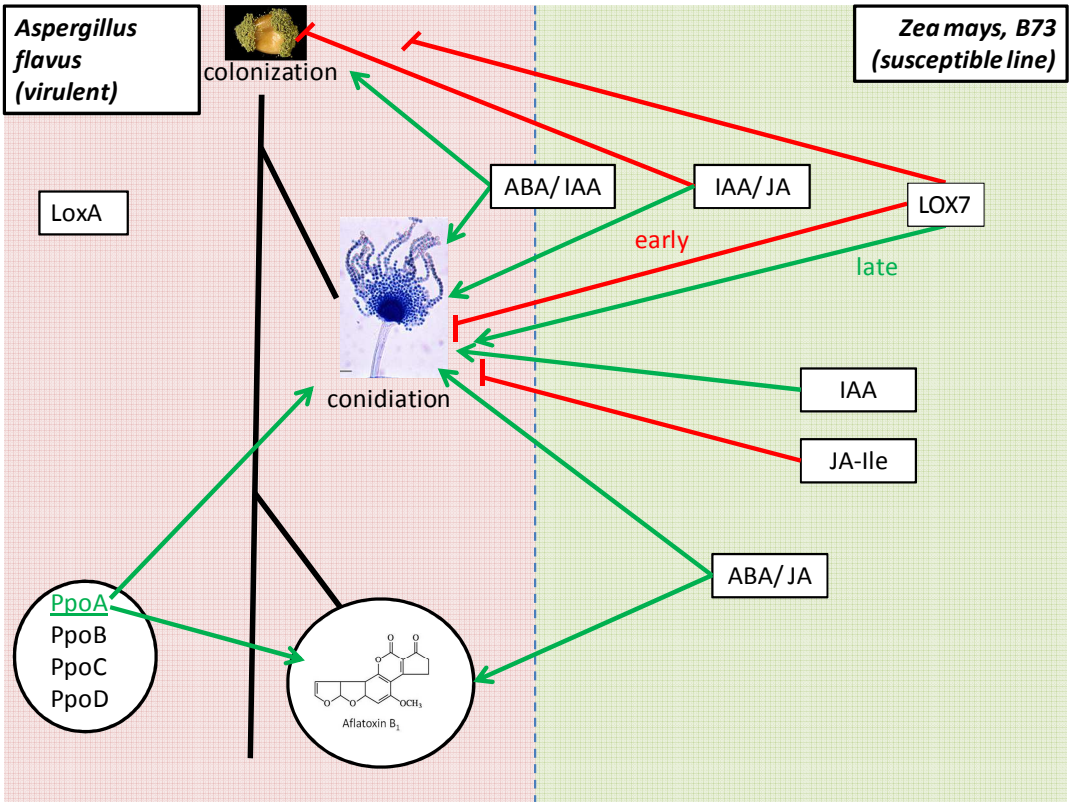

Fig. 27. Interaction between LOX7 and *A. flavus* fungal oxygenases during colonization, conidiation, and aflatoxin accumulation of maize seed. Fungal PpoA promotes conidiation and aflatoxin accumulation. LOX7 suppresses colonization, but has a temporal-specific regulation of conidiation. At early disease stages, LOX7 suppresses conidiation, but with disease progression, promotes conidiation. Hormone ratios of ABA/ IAA positively correlated with both colonization and conidiation. IAA/ JA negatively correlated with colonization but positively with conidiation. ABA/ JA positively correlated with conidiation and aflatoxin accumulation. Conidiation correlated with the positively with, IAA and negatively with JA-Ile. Green lines indicate positive regulation and red lines indicate negative regulation.

CHAPTER VI

CONCLUSION

Maize seeds respond to pathogen infection rapidly by both transcriptional and metabolic reprograming which eventually result in either activation of effective defense measures or by complete failure to respond in a timely manner, which leads to seed maceration of seed. One important defensive arsenal is the production of large group of lipid derived molecular signals, called oxylipins. Fungal pathogens produce oxylipins many of serve as signals to regulate many physiological processes including their ability to colonize seed, reproduce by means of spores, and produce secondary toxic metabolites called mycotoxins. Taken together our analysis tested an exciting hypothesis of a reciprocal oxylipin-mediated signal exchange between maize seed and *Aspergillus flavus* and summarized below (Fig. 28).

Despite belonging to separate classes, LOX3 and LOX7 were shown to function in general defense against *A. flavus* colonization. These genes make promising candidates for use in modern breeding techniques such as whole genome association studies and marker-assisted selection. At this time, little is known what is the nature and molecular and biochemical mechanisms behind the defense role of these two genes. Interestingly, both gene transcripts are strongly expressed in developing embryos (Fig. 10), the tissue most often colonized by *A. flavus* (Keller et al., 1994) and during the time window when *A. flavus* infects seeds via silk channel. It is possible, therefore, that the

13-LOX members, LOX7 and LOX10 exhibit a regulatory effect on conidiation, albeit, in opposite directions. LOX7 promotes normal conidiation while LOX10 has an inhibitory role. This suggests a certain level of specialization of these isoforms or their final oxylipin products.

Mutants perturbed in JA-biosynthesis displayed decreased aflatoxin accumulation, suggesting the involvement of JA production in the regulation of aflatoxin production. The results of this study provide a strong clue that JA alone is responsible for increased aflatoxin in kernels, yet the actual scenario appears more complex and requires input from other hormone signals. Consistently, the ratio of ABA/ JA correlated positively with aflatoxin accumulation. ABA and to a lesser known extent JA have long been considered the major drought stress hormones (Acharya and Assmann, 2009), my finding that ABA/JA ratio positively correlate with aflatoxin levels may fit well with well-documented fact that maize is more prone to be contaminated by aflatoxin under drought stress and high temperature conditions(Guo et al., 2008). This prompts a novel hypothesis that aflatoxin contamination of maize (and perhaps other oil-rich seed crops) may be more severe because of the plant stress response to drought by increasing ABA and JA signaling to allow plants to survive water limitations. With additional verification, the ABA/JA ratio may be used as a biochemical marker by maize breeders to screen lines for enhanced resistance to aflatoxin contamination.

Remarkably, amongst the Ppo gene family, I discovered that PpoA is specifically responsible for normal conidiation and aflatoxin biosynthesis. In addition, it is clear that collective Ppo genes appear to function together to suppress expression of LOX3,

suggesting that the combined mixture of oxylipin products from the lone LOX and the four Ppo isoforms are required to suppress host defense mechanisms against *A. flavus*.

This work provides a foundation for the understanding of oxylipin-mediated cross-talk between maize seed and mycotoxigenic fungi. The techniques developed through this work allow for a fast and simultaneous assessment of several fungal disease parameters and host phytohormone and expressional analysis. We expect this knowledge and techniques will expedite the studies of seed-fungal pathogen interactions and provide maize industry with novel targets for their breeding programs.

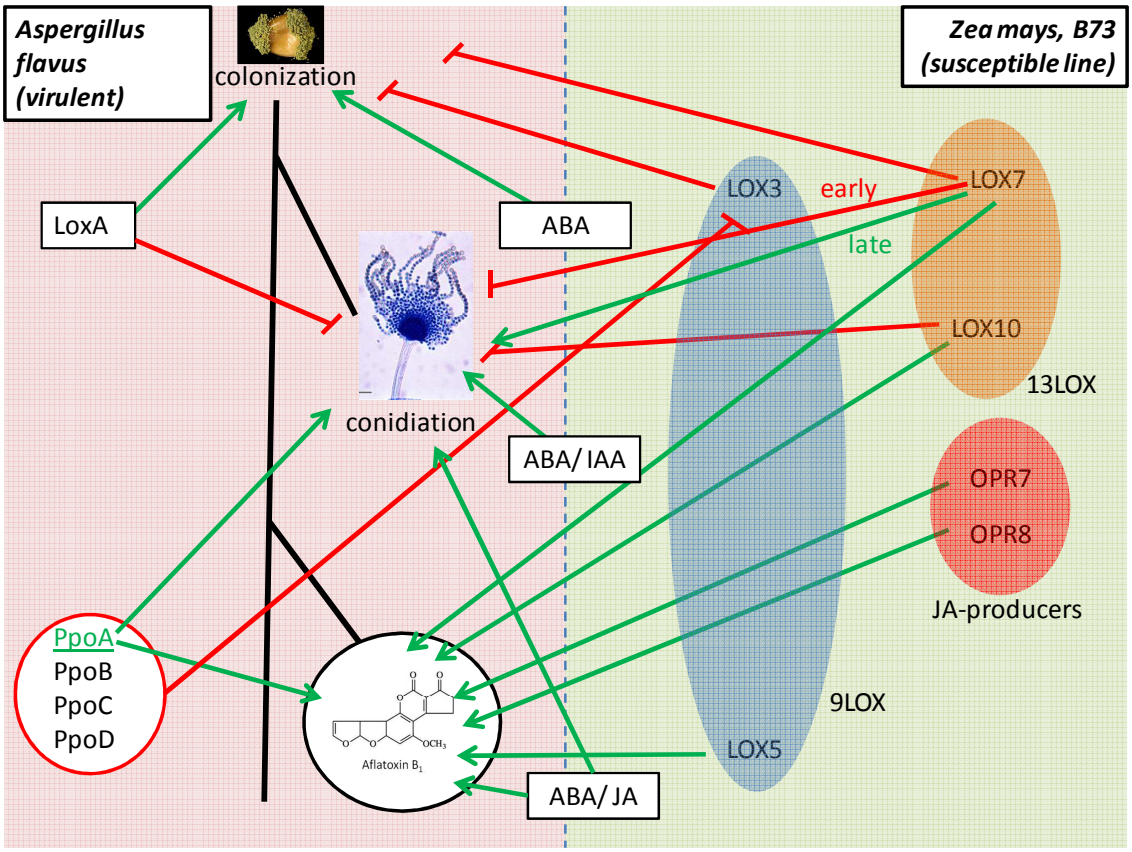

Fig. 28. Overview model depicting oxylipin-mediated communication between maize and *A. flavus.* Oxylipin-mediated signals from both plant host and fungal pathogen regulate *A. flavus* colonization, conidiation, and aflatoxin accumulation on maize kernels. Fungal-derived oxylipins from the lone AfLoxA positively promotes colonization but negatively regulates conidiation, while PpoA specifically promotes conidiation and aflatoxin accumulation. Multiple Ppo genes function together to negatively suppress the expression of maize LOX3 during seed infection. Plant-derived oxylipins from maize LOX3 and LOX7 are required for defense against *A. flavus* colonization. LOX10-derived products negatively regulate conidiation on maize seed. Maize oxylipin-mediated signals from LOX5, LOX7, LOX10, OPR7, and OPR8 induce aflatoxin accumulation. Hormone composition correlates with colonization, conidiation, and aflatoxin accumulation; abscisic acid (ABA) positively correlates with colonization, the ratio between ABA/ IAA positively correlates with conidiation, and the ratio of ABA/ JA positively correlates with conidiation and aflatoxin accumulation. Green lines indicate a positive interaction while red lines indicate a negative interaction.

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