Inactivation of the Lipoxygenase *ZmLOX3* Increases Susceptibility of Maize to *Aspergillus* spp.

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Plant and fungal lipoxygenases (LOX) catalyze the oxidation of polyunsaturated fatty acids, creating fatty-acid hydroperoxides (oxylipins). Fungal oxylipins are required for normal fungal development and secondary metabolism, and plant host-derived oxylipins interfere with these processes in fungi, presumably by signal mimicry. The maize LOX gene *ZmLOX3* has been implicated previously in seed-*Aspergillus* interactions, so we tested the interactions of a mutant maize line (*lox3-4*, in which *ZmLOX3* is disrupted) with the mycotoxigenic seed-infecting fungi *Aspergillus flavus* and *Aspergillus nidulans*. The *lox3-4* mutant was more susceptible than wild-type maize to both *Aspergillus* species. All strains of *A. flavus* and *A. nidulans* produced more conidia and aflatoxin (or the precursor sterigmatocystin) on *lox3-4* kernels than on wild-type kernels, in vitro and under field conditions. Although oxylipins did not differ detectably between *A. flavus*-infected kernels of the *lox3-4* and wild-type (WT) maize, oxylipin precursors (free fatty acids) and a downstream metabolite (jasmonic acid) accumulated to greater levels in *lox3-4* than in WT kernels. The increased resistance of the *lox3-4* mutant to other fungal pathogens (*Fusarium*, *Colletotrichum*, *Cochliobolus*, and *Exserohilum* spp.) is in sharp contrast to results described herein for *Aspergillus* spp., suggesting that outcomes of LOX-governed host-pathogen interactions are pathogen-specific.

Additional keywords: 9S-HPODE, *Fusarium verticillioides*.

One of the most economically important problems of cereal production worldwide is the contamination of seed by mycotoxins produced by seed-infecting fungi. These include the genera *Aspergillus* and *Fusarium*, whose members are serious pathogens of developing seeds as well as stored grain. These

fungi not only reduce grain integrity, but they also contaminate seed with mycotoxins, such as the aflatoxin (AF) produced by *Aspergillus flavus* and *A. parasiticus* and fumonisin by *Fusarium verticillioides*. Arguably, the crop most seriously afflicted by mycotoxigenic fungi is maize, which is frequently contaminated with both of these toxins.

Aspergilli primarily colonize and contaminate the lipid rich tissues of the host seed, such as the embryo and aleurone (Brown et al. 1993; Keller et al. 1994). At least two mechanisms may contribute to the lipid-AF connection. First, some plant fatty acids may be metabolized through mitochondrial and peroxisomal β -oxidation in the fungus, providing acyl-CoA precursors necessary for AF production (Maggio-Hall et al. 2005). In particular, oleic acid (a C18 monounsaturated fatty acid) induces proliferation of peroxisomes, thus increasing β -oxidation and subsequent AF production. Another hypothesis for the increased AF contamination of these seed tissues is that host-derived, oxidized lipids function as signal molecules that stimulate AF production in the fungus (Brodhagen and Keller 2006). C18- and C16-polyunsaturated fatty acids, particularly linolenic (C18:3) and linoleic (C18:2) fatty acids, can be oxidized by plant lipoxygenases (LOX) either at position 9 or 13 of their carbon chains (Feussner and Wasternack 2002). Because most distinct LOX isoforms preferentially catalyze either one or the other reaction, they are referred to as 9-LOX or 13-LOX. LOX primary products (fatty-acid hydroperoxides) are rapidly converted into a large class of oxygenated polyenoic fatty acids, collectively called oxylipins, via at least six separate biosynthetic enzymatic pathways (Feussner and Wasternack 2002; Porta and Rocha-Sosa 2002). Accumulating evidence suggests diverse physiological functions of oxylipins in plants, such as growth and development, resistance to insects and pathogens, and tolerance to salt, drought, and cold (Howe and Schilmiller 2002). For example, jasmonic acid (JA) and other 13-LOX-derived hydroperoxide products function as signals that regulate expression of defense-related and developmental genes (Turner et al. 2002).

Besides their role as endogenous signals in plant defense and development, host oxylipins have recently emerged as important signals in cross-kingdom communication between plants and pathogenic fungi (Brodhagen et al. 2008; Gao and Kolomiets in press; Tsitsigiannis and Keller 2007). The original evidence for this idea was obtained from bioassays showing that the primary products of plant 13- and 9-LOX (13S-hydroperoxyoctadecadienoic acid [13S-HPODE] and 9S-HPODE, respectively) modulate fungal developmental processes in-

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cluding vegetative growth and sporulation. Moreover, 13S-HPODE and 9S-HPODE altered the production of AF and other secondary metabolites by *Aspergillus* spp. (Burow et al. 1997; Calvo et al. 1999).

The physiological effects of plant-derived oxylipins on *Aspergillus* spp. are often attributed to mimicry by 13S-HPODE and 9S-HPODE of structurally similar fungal oxylipins (Tsitsigiannis and Keller 2006). *A. nidulans* produces a group of oxylipins, collectively named psi factor (for precocious sexual inducer), via the enzymatic action of the dioxygenases PpoA, PpoB, and PpoC (Champe and El-Zayat 1989; Champe et al. 1987; Mazur et al. 1990, 1991; Tsitsigiannis et al. 2004a and b). Specific oxylipins that comprise psi factor have differing (and sometimes synergistic) effects on the ratio of sexual to asexual spore production (Champe and El-Zayat 1989; Champe et al. 1987; Tsitsigiannis et al. 2004a and b, 2005), the ability of the fungus to colonize host seed, and the production of secondary metabolites (Tsitsigiannis and Keller 2006). Among the secondary metabolites affected is sterigmatocystin (ST), the penultimate precursor to AF in *A. flavus* and *A. parasiticus* but the final product of the biosynthetic pathway in *A. nidulans*. Treatment of *Aspergillus* cultures with the pure plant oxylipin 9S-HPODE resulted in increased conidiation (Calvo et al. 1999) and AF production as well as accumulation of a ST biosynthetic gene transcript (Burow et al. 1997). We therefore hypothesized that 9S-HPODE, produced by 9-LOX enzymes, might be a susceptibility factor in maize for AF contamination.

9S-HPODE may potentially be produced in maize tissues by six different putative maize 9-LOX (M. Kolomiets, *unpublished results*). Expression of the gene for one of these, *ZmLOX3*, occurs in the developing embryos (Gao et al. 2007) and is further upregulated during kernel infection by both *A. flavus* and *F. verticillioides* (Wilson et al. 2001). Therefore, we predicted that disruption of *ZmLOX3* might decrease sporulation and AF production. We previously generated a mutated *ZmLOX3* allele called *lox3-4* by insertion of the transposable element *Mutator* into the *ZmLOX3* coding sequence. The *lox3-4* line was more resistant than the wild-type (WT) to *F. verticillioides*; infected *lox3-4* kernels yielded twofold fewer conidia than infected near-isogenic WT kernels (Gao et al. 2007). Furthermore, *F. verticillioides* produced 100- to 200-fold more fumonisin B1 in the WT kernels than in the *lox3-4* kernels. In addition to *F. verticillioides*, *lox3-4* mutant was more resistant to two foliar pathogens, *Colletotrichum graminicola* and *Cochliobolus heterostrophus* (Gao et al. 2007) as well as to the root-rotting pathogen *Exserohilum pedicellatum* (Isakeit et al. 2007). Collectively, these data provided strong evidence that a single genetic element, the functional *ZmLOX3* gene, contributes to the susceptibility of maize to diverse fungal pathogens.

The present study examined the role of *ZmLOX3* in maize interactions with AF-producing *A. flavus* and ST-producing *A. nidulans*. In sharp contrast to the results obtained with the other fungal pathogens, conidiation and toxin production were increased during colonization of the *lox3-4* mutant in both laboratory and field conditions. Analysis of the lipids in the infected seed suggested that changes in fatty-acid levels rather than changes in levels of oxylipins themselves could underlie the increased growth and toxin biosynthesis of these *Aspergillus* species on the *lox3-4* mutant.

RESULTS

Conidia and AF production by *A. flavus* are increased on *lox3-4* mutant.

To test our hypothesis that disruption of *ZmLOX3* might decrease sporulation and AF production, we performed kernel

assays on the knockout *lox3-4* mutant and near-isogenic WT maize. As Figure 1A illustrates, *lox3-4* mutant kernels were colonized by *A. flavus* NRRL 3357 to visibly greater levels compared with the WT. Similar results were obtained when we infected kernels with *A. flavus* 12S (data not shown). Consistent with the increase in fungal growth, the *lox3-4* mutant kernels supported production of significantly more conidia at 3.5 and 7 days postinfection (dpi). We observed similar results whether we infected with *A. flavus* NRRL 3357 (Fig. 1B) or *A. flavus* 12S (data not shown). Although attempts to measure AF from *A. flavus* 12S-infected kernels were unsuccessful, we observed that maize *lox3-4* kernels infected with *A. flavus* NRRL 3357 produced significantly more AF than WT kernels at both 3.5 and 7 dpi (Fig. 1C).

Fig. 1. The *lox3-4* mutant permits greater colonization, conidiation and aflatoxin (AF) production by *Aspergillus flavus* than the wild type (WT) in kernel assays. **A**, Comparison of visible colonization by *A. flavus* NRRL 3357-infected kernels. WT = *A. flavus*-infected wild type kernels, *lox3-4* = *A. flavus*-infected *lox3-4* kernels. The pictures were taken at 7 days post-inoculation (dpi). Results were similar for kernels infected with *A. flavus* 12S (data not shown). **B**, Conidiation by *A. flavus* NRRL 3357 following infection of *lox3-4* mutant and WT maize kernels. Similar results were obtained when kernels were infected with *A. flavus* 12S (data not shown). **C**, AF production by *A. flavus* NRRL 3357 on *lox3-4* mutant and WT maize kernels. Error bars denote one standard error. Different letters above bars denote significantly different means ($P < 0.05$, analysis of variance) between WT and mutant within same timepoint.

AF accumulation and *Aspergillus* ear rot on the *lox3-4* mutant in the field.

Next, we tested whether the *lox3-4* mutant would support greater fungal colonization and AF production under field conditions as in kernel assays. In spring of 2005 and 2006, seeds from near-isogenic lines (NIL) were planted in two separate Texas locations, Weslaco and College Station. In addition to any naturally existing disease pressure, the corn ears of both genotypes were inoculated 10 days after mid-silking with conidia from the *A. flavus* NRRL 3357 strain. AF production and ear rot disease severity were examined after harvest.

AF production. AF accumulation was greater in the *lox3-4* mutant than in the WT in both years and at both locations (Fig. 2). The differences were significantly greater in inoculated ears in 2005 at College Station ($P < 0.05$) and Weslaco ($P < 0.01$) and in 2006 at Weslaco ($P < 0.05$). In 2006 at College Station, the *lox3-4* mutant accumulated about 1.5 times more AF than the WT, but the difference was not statistically significant. Even in the noninoculated control ears (which were naturally infected by indigenous *Aspergillus* spp.), the *lox3-4* mutant accumulated up to 10-fold more AF than the WT NIL; at the Weslaco site, these differences were statistically significant in both 2005 and 2006 (e.g., at Weslaco in 2006, there were 460 ppb AF on *lox3-4* kernels vs. 46 ppb AF on WT kernels, $P < 0.05$). At the College Station site, the differences in AF accumulation from incidental *Aspergillus* infection on uninoculated *lox3-4* and WT ears were not significant (data not shown).

Ear rot incidence and disease severity. At College Station in both 2005 and 2006, AF contamination was significantly correlated with *Aspergillus* ear rot severity and incidence ($r = 0.857$, $P < 0.001$ and $r = 0.533$, $P < 0.05$, respectively, in 2005; and $r = 0.517$, $P < 0.05$ and $r = 0.808$, $P < 0.001$, respectively, in 2006). In 2005 at College Station, the difference in incidence of ear rot caused by *A. flavus* between the two genotypes (39% for the *lox3-4* mutant and 24% for the WT) was not statistically significant ($P > 0.05$), but the severity of ear rot was significantly greater ($P < 0.05$) for the *lox3-4* mutant (9.3%) than for the WT (1.2%). In 2006 at College Station, the incidence of ear rot was 54% for the *lox3-4* mutant and 27% for the WT; however, this difference was not statistically significant ($P > 0.05$). In 2006, the severity of ear rot caused by *A. flavus* in both locations was 1% or less and there were no evident differences between the genotypes at either location. There was no correlation of AF with ear rot in Weslaco in either year. In 2005 and 2006, the incidence of ear rot in Weslaco was less than 1% for both genotypes and there were no evident differences between the genotypes ($P > 0.05$).

Lipid profiling in *A. flavus*- and

F. verticillioides-infected *lox3-4* mutant and WT kernels.

To investigate whether specific oxylipins or their fatty-acid precursors were associated with the increased susceptibility of the mutant to *A. flavus*, we measured levels of 42 different oxylipins and free fatty acids in the *lox3-4* mutant and WT kernels after infection with *A. flavus* 12S. Of these, four free fatty-acid oxylipin precursors, palmitic (C16:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids, were significantly increased in *lox3-4* mutant but not in WT kernels infected with *A. flavus* at 72 hpi ($P < 0.05$) (Fig. 3A through C). Among the 9-LOX- or 13-LOX-derived oxylipins measured in this study, a difference between concentrations in the mutant and the WT was observed only for JA, one of the biologically active products of the 13-LOX-derived octadecanoid biosynthetic pathway. As Figure 3D shows, JA concentrations increased approximately threefold ($P < 0.05$) in response to *A. flavus* infection in the *lox3-4* mutant kernels. Although WT kernels responded to the infection by increased production of JA, this increase was not statistically significant.

Because our previous study showed that the *lox3-4* mutant was more resistant to contamination with fumonisin B1 and supported production of fewer *F. verticillioides* conidia (Gao et al. 2007), we tested whether the fatty-acid content of *F. verticillioides*-infected kernels differed from that observed in *A. flavus*-infected kernels. In contrast to the observations described above for *A. flavus*-infected kernels, it was WT *F. verticillioides*-infected kernels not the *lox3-4* mutant kernels that exhibited increased fatty acids. Specifically, C16:0, C18:0, C18:1 (including both 9Z and 11Z isomers), C18:2, and C18:3 fatty acids were increased following infection of WT but not *lox3-4* mutant kernels with *F. verticillioides*. This increase in certain fatty acids could not be detected by 3.5 dpi but was observed later at 7 dpi (Fig. 4). Compared with noninfected seed (0 dpi), the levels of induction were the strongest for three of the most abundant seed fatty acids, C16:0, C18:1 (9Z), and C18:2 (with induction levels averaging 1.8 $\times$, 3.1 $\times$, and 2.5 $\times$, respectively). Importantly, no difference in the fatty-acid levels was observed between the mutant and WT in the noninfected, dry seed (0 dpi), suggesting that *ZmLOX3* exerts its effect on the fatty-acid composition and seed-pathogen interactions only in response to infection. These results can be explained by the fact that *ZmLOX3* expression is not detectable in dry, nongerminating seed but is induced highly by pathogen infection (Wilson et al. 2001). Unlike in the *A. flavus*-infected kernels, JA levels did not differ between the mutant and WT at any timepoints after *F. verticillioides* infection (data not shown).

Fig. 2. The *lox3-4* mutant accumulated significantly higher levels of aflatoxin in the field. **A**, Weslaco 2005; **B**, College Station 2005; **C**, Weslaco 2006; and **D**, College Station 2006. Values are the means of four replicates of 8 to 12 cobs each. Asterisks above the columns indicate significant differences between genotypes (* denotes $P < 0.05$, ** denotes $P < 0.01$, analysis of variance). Log-transformed values were used for the analysis. Untransformed means are presented.

Sporogenesis and mycotoxin production by *Aspergillus nidulans* oxylipin mutants.

Because an earlier study showed that *A. nidulans* oxylipin mutants were aberrant in seed colonization of WT maize and peanut (Tsitsigiannis and Keller 2006), we next investigated whether these fungal oxylipin mutants were affected in their ability to colonize seed or to produce spores and mycotoxin when grown on the *lox3-4* mutant. Specifically, we compared the spore and ST production on maize WT and *lox3-4* kernels of five *A. nidulans* strains, including WT, $\Delta ppoB$, $\Delta ppoAC$, and two strains heterologously expressing the *ZmLOX3* gene (Table 1). Regardless of the specific *A. nidulans* oxylipin genotype, the *lox3-4* kernels always supported more fungal growth than did WT kernels (Fig. 5A). This was reflected in the increased number of conidia produced on the *lox3-4* mutant compared with WT kernels by all fungal strains (Fig. 5B).

As previously observed in culture and on a different line of WT maize (Table 2), the $\Delta ppoB$ mutant produced more conidia and the double $\Delta ppoAC$ mutant fewer conidia than the WT *A. nidulans* when grown on WT kernels. This trend was conserved on the *lox3-4* mutant kernels. Addition of the *ZmLOX3* gene into *A. nidulans* permitted still greater conidiation on *lox3-4* seeds ($P < 0.05$). Finally, $\Delta ppoAC$ mutants, which have been shown previously to produce more sexual spores than WT fungus when grown on synthetic medium (Table 2), also produced more sexual spores on the *lox3-4* mutant than on WT kernels (data not shown).

ST production was also higher on the *lox3-4* kernels than on the WT, regardless of *A. nidulans* strain (Fig. 6A). However, the ST production levels varied among fungal strains. ST production on the *lox3-4* mutant kernels was greatest in both the *ZmLOX3*-expressing *A. nidulans* strains (Fig. 6A and B). This correlated with conidial production (Fig. 5A). The greatest enhancement in ST production on the *lox3-4* kernels was by $\Delta ppoAC$, a strain barely able to synthesize ST on WT kernels (Fig. 6A and B) (Tsitsigiannis and Keller 2006).

DISCUSSION

A general role for a maize 9-LOX in susceptibility to fungal pathogens appeared to be supported by two recent studies showing that loss of the 9-LOX gene *ZmLOX3* resulted in decreased fumonisin production by the mycotoxigenic maize pathogen *F. verticillioides* (Gao et al. 2007) and that expression of this same gene remediated ST production in an *A. nidulans* oxylipin mutant (Brodhagen et al. 2008). However, the current study shows that the *lox3-4* mutant maize is more susceptible to *Aspergillus* growth and to AF and ST contamination under both field and kernel assay conditions. Because one unexpected result was the lack of a difference in 9S-HPODE between *A. flavus*-infected WT and *lox3-4* kernels, possible insights and caveats of the lipid profiles are first discussed.

Our previous studies involving the *lox3-4* mutant suggest that disruption of *ZmLOX3* resulted in altered oxylipin profiles only in the tissues or treatments in which the WT *ZmLOX3* gene would normally be expressed, either constitutively or in

an inducible manner. For instance, when oxylipins in healthy (uninfected) 4-day-old germinating embryos of WT and *lox3-4* mutant kernels were profiled, 9-LOX-derived oxylipins were lower in the *lox3-4* mutant seeds (Gao et al. 2007). Four-day-

Fig. 3. Contents of selected free fatty acids and jasmonic acid (JA) in *Aspergillus flavus* 12S-infected *lox3-4* and wild type (WT) kernels. Differences were observed in **A**, palmitic (16:0) and oleic (18:1), **B**, linoleic (18:2), and **C**, linolenic (18:3) acid contents, as well as in **D**, JA contents. At least four replicates comprising at least four inoculated kernels each were measured 72 h postinfection. Error bars denote one standard error. Letters above bars denote significant differences ($P < 0.05$, analysis of variance) between treatments within each genotype.

old germinating kernels were used in that study to quantify oxylipins because, at this developmental stage, *ZmLOX3* transcripts accumulated to the highest levels (Gao et al. 2007; Wilson et al. 2001). Inactivation of *ZmLOX3* also affected oxylipin profiles of the nonseed tissues that express this gene constitutively; the levels of 9-LOX-derived oxylipins were

lower but JA levels were higher in healthy roots of the *lox3-4* mutant than in the WT (Gao et al. 2008). No difference was observed in the oxylipin levels between the untreated mutant and WT leaves, a tissue in which *ZmLOX3* transcripts are not detectable. *ZmLOX3* was also not expressed in either untreated dry seed or in control mock-inoculated but nongerminating seed.

Fig. 4. Contents of selected free fatty acids in *Fusarium verticillioides*-infected *lox3-4* and wild type (WT) kernels. 16:0 = palmitic acid, 18:0 = stearic acid; 18:1 = oleic acid, 18:2 = linoleic acid, and 18:3 = linolenic acid. The data are expressed as means $\pm$ standard deviation ($n = 2$); dpi = days postinfection.

Table 1. *Aspergillus flavus* and *A. nidulans* strains used in this study

Strains	Genotype	Genotype description	Source
<i>A. flavus</i> 12S	Wild type (WT)	Wild type	Calvo et al. 1999
<i>A. flavus</i> NRRL 3357	WT	Wild type	Wicklow et al. 1998
RDIT9.32	WT	<i>A. nidulans</i> <i>veA</i> (wild type)	Tsitsigiannis et al. 2004b
RDIT59.1	$\Delta ppoB$	<i>A. nidulans</i> $\Delta ppoB::pyroA$ $pyroA4$ <i>veA</i>	Tsitsigiannis et al. 2005
RDIT54.7	$\Delta ppoAC$	<i>A. nidulans</i> $\Delta ppoA::metG$ $\Delta ppoC::trpC$ <i>metG1</i> <i>trpC801</i> <i>veA</i>	Tsitsigiannis et al. 2004a
RDIT106.5	$\Delta ppoAC+ZmLOX3$	<i>A. nidulans</i> $\Delta ppoA::metG$ $\Delta ppoC::trpC$ <i>gpdA(p)::ZmLOX3::pyroA</i> <i>veA</i>	Brodhagen et al. 2008
RDIT98.5	WT+ <i>ZmLOX3</i>	<i>A. nidulans</i> <i>gpdA(p)::ZmLOX3::pyroA</i> <i>veA</i>	Brodhagen et al. 2008

However, *ZmLOX3* was induced in *A. flavus*-infected kernels (Wilson et al. 2001). Thus, 9-LOX-derived oxylipins were not expected to change in nongerminating kernels but were expected to be lower in *lox3-4* mutant than WT kernels after *A. flavus* attack. However, no significant differences in 9-LOX-derived oxylipins between WT and *lox3-4* kernels were observed even after *A. flavus* infection (data not shown). The only difference in oxylipins was observed in kernels infected with *A. flavus*, in which JA, a 13-LOX derivative, was higher in the *lox3-4* mutant than in the WT (Fig. 3D). These observations underscore the importance of kernel phenology on the overall influence of *ZmLOX3* on the oxylipin profile as well as the role of pathogen attack. Because *Aspergillus* infection is known to alter expression of several LOX genes in maize kernels (Wilson et al. 2001), it is possible that any differences in oxylipin profiles between WT and *lox3-4* kernels infected with *A. flavus* may have been overshadowed by upregulation of LOX genes other than *ZmLOX3* during the defense response. Because maize harbors several 9-LOX genes, it is possible that the expression of other

9-LOX genes were upregulated in ungerminated seeds to compensate for the missing *ZmLOX3*. For example, in maize root tissues, expression of *ZmLOX5* (a 9-LOX) and *ZmLOX10* (a 13-LOX) are normally not constitutive but are inducible (e.g., during nematode attack). However, in the *lox3-4* mutant roots, both *ZmLOX5* and *ZmLOX10* were constitutively expressed (Gao et al. 2008). Similar compensatory mechanisms were shown for *A. nidulans*, in which loss of individual *ppo* genes causes altered expression of the remaining *ppo* genes (Tsitsigiannis et al. 2005). Finally, our lipid profiles crudely averaged overall kernel lipid composition and would not have detected localized differences within a seed in which oxylipin production might be very low or very high (e.g., in cells responding to pathogen attack). Thus, oxylipin levels may indeed vary in the embryonic maize tissues of infected *lox3-4* seed at a finer level of resolution than detected in this study, and such differences might be locally important to a germinating fungal spore or intruding hyphae.

Although our methods did not reveal any detectable differences in levels of 9S-HPODE and 13S-HPODE, there were

Fig. 5. Phenotypes of *Aspergillus nidulans* oxylipin mutants growing on maize (wild type) WT and *lox3-4* mutant kernels. Comparisons were made between WT and *lox3-4* mutant kernels inoculated with various *A. nidulans* strains, regarding **A**, visual differences in fungal growth and **B**, production of asexual spores. Conidia were counted and pictures were taken at 7 days postinfection. Five replicates of four kernels each were compared. Error bars represent one standard error. Letters above bars denote significant differences ($P < 0.05$, analysis of variance) between WT and *lox3-4* mutant kernels infected with the same *A. nidulans* strain.

Table 2. Overview of phenotypes of *Aspergillus nidulans* wild type (WT) and oxylipin mutants

Genotype	Asexual spores			Sexual spores			Sterigmatocystin		
	Agar ^a	Peanut ^b	Maize	Agar ^a	Peanut ^b	Maize	Agar	Peanut ^c	Maize
WT	++	+++	+++	++	(25)+ ^d	ND	+++	+	++
$\Delta ppoA$	+++	+++++	ND	+	(25)+	ND	+	++	ND
$\Delta ppoB$	+++++	+++++	++++	+	(25)+	ND	+++++	++	+++++
$\Delta ppoC$	+	++	ND	+++	(12)+	ND	++++	++	ND
$\Delta ppoAC$	+	+	++	+++++	+	ND	-	-	+
$\Delta ppoABC$	+	+	ND	(12)+	+	ND	-	-	-
WT + <i>ZmLOX3</i>	+++ ^e	ND	+++	++ ^e	ND	ND	(9)+	ND	(8)+
$\Delta ppoAC$ + <i>ZmLOX3</i>	++ ^e	ND	+++	++ ^e	ND	ND	++++	ND	(8)+

^a Except where noted, data adapted from Tsitsigiannis and associates (2005). Proportions are relative within, but not across, columns. ND = not determined.

^b Except where noted, proportions derived from Tsitsigiannis and Keller (2006).

^c Except where noted, proportions estimated visually from Tsitsigiannis and Keller (2006).

^d Indicates number of '+' symbols.

^e Proportions derived from Brodhagen and associates (2008).

Fig. 6. Sterigmatocystin (ST) production by *Aspergillus nidulans* wild type (WT) and Δppo mutants after inoculation onto *lox3-4* and WT kernels at 3 and 7 days postinfection (dpi). ST was separated from crude extracts by thin-layer chromatography and was quantified by densitometry. **A**, Effect of maize *ZmLOX3* deletion. Sections from thin-layer chromatograms, demonstrating differences in ST production by *A. nidulans* strains grown on WT or *lox3-4* mutant kernels at 7 dpi. The numbers on the right denote ratios (*lox3-4*/WT) of ST production in infected kernels as measured by densitometry. Values shown are the means of four replicates $\pm$ one standard deviation. **B**, Effect of *A. nidulans* Δppo deletions. ST production at 3 and 7 dpi by WT *A. nidulans* and Δppo mutants grown on maize WT and *lox3-4* kernels. Numbers above bars denote the ratios of ST production (Δppo mutant/WT) of *A. nidulans* within each maize genotype. Values shown are the means of four replicates of five kernels each. Asterisks above bars represent significant differences in ST production for each fungal genotype between WT and *lox3-4* mutant kernels ($P < 0.05$). ST, sterigmatocystin standard. AU = absorbance unit.

significant differences in free fatty-acid levels in the *lox3-4* kernels versus WT kernels. *A. flavus*-infected *lox3-4* kernels were increased in palmitic (C16:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acid content (Fig. 3A through C). Like 9S-HPODE, oleic and linoleic acid have been shown to induce *Aspergillus* conidiation (Calvo et al. 1999) and AF and ST biosynthesis (Burow et al. 1997; Maggio Hall et al. 2005). Oleic acid in particular can be a powerful stimulant of ST and AF production, presumably due to an indirect effect on ST and AF precursor abundance. Oleic acid enhances the proliferation of peroxisomes, one of the organellar locations where β -oxidation of fatty acids occurs in *Aspergillus* spp. (Maggio Hall et al. 2005). β -oxidation metabolizes fatty acids into abundant pools of acyl-CoA, which is required for AF or ST polyketide biosynthesis in the aspergilli. β -Oxidation mutants of *A. nidulans* were defective in ST production in oleic acid-amended culture medium as well as on maize seed (Maggio-Hall et al. 2005). Furthermore, field studies have shown that peanut seeds with high oleic-acid content are more heavily contaminated with AF (Xue et al. 2003). Thus, it is possible that the increased oleic-acid content of the infected *lox3-4* kernels contributed to the higher ST and AF production by the fungi.

To examine the interplay of maize and *Aspergillus* oxylipin signaling, WT and *lox3-4* seeds were inoculated with *A. nidulans* WT and known oxylipin mutants. The phenotype of each *A. nidulans* strain reflects its unique oxylipin profile (with which the host oxylipin profile presumably blends). Notably, a *AppoAC* mutant of *A. nidulans*, which produces no detectable ST in culture and little on seed, produced near-WT levels of the mycotoxin on *lox3-4* mutant maize kernels. This observation suggests that one or more unknown factors in the mutant kernels compensates for the missing fungal oxylipins (for example, the increased oleic-acid levels of *Aspergillus*-infected *lox3-4* kernels). In seeming contradiction, expression of *ZmLOX3* in the *AppoAC* mutant also restores ST production (Brodhagen et al. 2008). This suggests that separate regulatory mechanisms connect *ZmLOX3* to ST production when the gene is expressed in maize versus fungal tissues. Because the *lox3-4* kernels supported greater conidiation, regardless of *Aspergillus ppo* genotype, than did WT kernels, addition of the *ZmLOX3* gene back into the *Aspergillus*-seed pathosystem (via expression in the fungus) was expected to complement the effect of the *lox3-4* mutation by lowering conidial production. Instead, *A. nidulans* expressing *ZmLOX3* produced the most conidia of any genotype on *lox3-4* seeds. The latter result may reflect a synergy between high oleic acid in the *lox3-4* seeds and *ZmLOX3* in the fungus, both of which, by themselves, have a positive effect on ST production (Brodhagen et al. 2008; Maggio-Hall et al. 2005). Alternatively, *ZmLOX3* expressed in the fungus may simply reflect a simpler signal in the fungus (a single 9-LOX and its product) than in the plant, where expression or loss of *ZmLOX3* occurs in the context of many synergistic, opposing, or compensatory LOX and a complex array of downstream derivatives from the oxylipin biosynthetic pathways. The solo *ZmLOX3* expressed in the fungus reflects the bioassays in which pure 9S-HPODE was added to fungal cultures; the phenotype resulting from *lox3-4* deletion in host kernels is apparently and predictably less straightforward.

This work reveals that *A. flavus* and *F. verticillioides*, two prevalent maize seed pathogens, respond oppositely to the deletion of a maize 9-LOX gene. Multiple lines of evidence from previous experiments pointed to 9-LOX activity as a susceptibility factor in the maize-*Aspergillus* interaction (Tsitsigiannis and Keller 2006). Inactivation of *ZmLOX3* in maize revealed that the relationship is more complex than we hypothesized, as *lox3-4* mutants are more susceptible to *Aspergillus* colonization and AF

accumulation. By contrast, although *F. verticillioides* is able to infect the *lox3-4* mutant kernels similarly to the WT, fumonisin and microconidia production in the mutant seed is dramatically decreased (Gao et al. 2007). One possible explanation for the different phenotypes of the two fungi on *lox3-4* mutant maize kernels is their ability to induce fatty-acid accumulation either in the mutant or WT. In contrast to the increased fatty-acid content in *A. flavus*-infected *lox3-4* mutant kernels, it was the WT seed and not the mutant that responded to *F. verticillioides* infection by increased production of all fatty acids tested. Clearly, additional experimentation will be required to elucidate biological relevance of the fatty acids on the differential behavior of the two fungi on the *lox3-4* mutant seed.

An opposite ability to suppress either *Fusarium* spp. or *Aspergillus* spp. (but not both) also has been observed on maize bearing mutations in other LOX genes (data not shown), suggesting that, while individual LOX genes contribute uniquely to pathogen susceptibility, the outcomes of oxylipin-governed host-pathogen interactions are pathogen-specific. This phenomenon is critical from a practical standpoint; because *Aspergillus* spp. and *Fusarium* spp. coexist naturally in maize fields, engineered resistance to one pathogen at the expense of susceptibility to the other is useless.

MATERIALS AND METHODS

Plant materials and fungal strains.

The generation of maize *lox3-4* mutants by *Mutator*-transposable element-insertional mutagenesis was described previously (Gao et al. 2007). The mutant and WT are NIL at the BC4F5 genetic stage in the B73 background.

Aspergillus flavus and *A. nidulans* strains used in this study are listed in Table 1. *A. flavus* strains were cultured on potato dextrose agar (Difco Laboratories, Detroit) at 29°C, and *A. nidulans* strains were cultured at 37°C on glucose minimal medium (Calvo et al. 2001). Cultures were grown inside cardboard boxes to achieve continuous dark.

Fusarium verticillioides 7600 (M3125; Fungal Genetics Stock Center, University of Missouri, Kansas City, KS, U.S.A.) was grown on potato dextrose agar (B&D, Sparks, MD, U.S.A.) at 28°C.

Kernel infection assays and spore enumeration.

To elucidate the effect of the *ZmLOX3* gene disruption on the production of conidia, we used *A. flavus* NRRL 3357 and five *A. nidulans* strains (Table 1) in a kernel assay. The *lox3-4* and WT kernels were surface-disinfested with 0.6% sodium hypochlorite for 10 min and were rinsed five times with distilled H₂O. To provide an infection court, the kernels were wounded by cutting the embryo side with a razor blade, with the cut depth of about 0.5 mm. Seeds were blotted dry, were placed in a 20-ml glass scintillation vial (Wheaton Science, Millville, NJ, U.S.A.), and were inoculated with 1×10^6 conidia suspended in 200 μ l of 0.001% Tween 20. Control seeds received an equal amount of 0.001% Tween 20. Inoculated or mock-inoculated kernels were used ($n = 4$ or 6 per biological replicate), with six replicates per experiment. The vials were kept in a box with wetted filter paper to form humidity chambers and were incubated at 29°C with 12-h light and dark cycles. Sterile distilled H₂O was added as needed to keep the filter paper base of the humidity chambers moist. Kernels were harvested at 3.5 and 7 dpi for spore counting (as described above) and quantification of AF or ST. For ST measurement, six infected kernels per replicate were frozen in liquid nitrogen until analysis. Five and six replicates were used for spore counting and ST measurement, respectively. The conidiation experiments were repeated at least four times with consistent results.

To determine the effect of the *ZmLOX3* gene disruption on the lipid composition of infected kernels and the production of conidia, we inoculated kernels of *A. flavus* 12S as described by Wilson and associates (2001). Three biological replicates, each containing six kernels of either the *lox3-4* or WT kernels, were inoculated by overlaying wounded embryos with 5 μ l of 0.001% (vol/vol in water) Tween 80 containing an estimated 5,000 conidia of *A. flavus* 12S. Control seeds were inoculated with pure 0.001% Tween 80. Kernels were harvested at 48 and 72 h postinfection (hpi) and were vortexed for 1 min in 0.001% Tween 80 to dislodge spores. The spore suspension was decanted, and spores were enumerated using a hemacytometer. Our isolate of *A. flavus* 12S did not produce AF reliably enough for accurate comparisons, so instead, *A. flavus* NRRL 3357 (Wicklowsky et al. 1998; Windham et al. 2003) was examined for AF production as described below. Spore enumeration was also performed on WT and *lox3-4* kernels infected as described below with *A. flavus* NRRL 3357, with results similar to those for *A. flavus* 12S (data not shown).

AF and ST quantification.

A. flavus NRRL 3357-infected seed were examined for AF content using monoclonal affinity columns and fluorescence determination with the Vicam Aflatest (Vicam, Watertown, MA, U.S.A.), according to the United States Department of Agriculture Federal Grain Inspection Service (USDA FGIS) protocol (United States Department of Agriculture 1997), with the following modification. Kernels of a known weight (0.5 to 1.3 g) were ground and extracted with 20 ml of 80% (vol/vol in water) methanol using a blender. Each treatment consisted of four biological replicates with four kernels per replicate.

For the ST analysis in planta, corn kernels were collected in 50-ml Falcon tubes and were vortexed for 1 min in 0.001% Tween 80 to dislodge spores. This suspension (still containing kernels) was extracted sequentially in acetone and then in chloroform, for a final ratio of 1:1:1 (vol/vol/vol) acetone/chloroform/0.001% Tween 80. Following each solvent addition, samples were shaken for 10 min at 150 rpm, allowed to stand for 5 min, and were centrifuged for 10 min at 3,000 rpm. The organic lower phase was collected, and the solvent was evaporated in a fume hood. The presence of abundant seed lipids in the samples hampered the clear observation of ST on thin-layer chromatography (TLC) plates at this stage of purification, so a second extraction was carried out as follows. Samples were resuspended in 5 ml of 0.1 M NaCl methanol/water [55:45 (vol/vol)] and 2.5 ml of hexane and were vortexed for 1 min. Samples were centrifuged at 2,000 rpm for 5 min. The hexane layer was collected, and the interphase layer (containing fatty acids) was discarded. The remaining aqueous phase was washed with an additional 2.5 ml of hexane. The hexane extracts were combined and allowed to evaporate. The residue was then resuspended in 500 μ l of hexane, 30 μ l of which was spotted onto silica gel TLC plates and separated in a hexane/ethyl acetate (40:10, vol/vol) solvent system. Quantification of ST spot intensity (detected at 245 nm) on the TLC plates was accomplished using a CAMAG II densitometer (CAMAG, Muttenz, Switzerland) equipped with CATS TLC software (version 3.20). Experiments were performed with four replications per treatment. All procedures were carried out at room temperature.

Profiling of oxylipins and fatty acids.

Quantification of oxylipins and fatty acids was conducted as described in Stumpe and associates (2005). For measurement of oxylipins and fatty acids, six kernels of *lox3-4* and near-isogenic WT were inoculated with conidia of *A. flavus* 12S and were incubated for 72 h at 29°C in darkness, as described

above. Four and six replicates were used for mock- and *A. flavus*-inoculated *lox3-4* or NIL WT with four kernels each, respectively. For oxylipin and fatty-acid profiling of *Fusarium verticillioides*-inoculated kernels, the kernel assay was conducted similarly to that of *A. flavus*. Briefly, the *lox3-4* and WT kernels were inoculated with 1×10^6 conidia suspended in 200 μ l of 0.001% Tween 20. Control seeds received an equal amount of 0.001% Tween 20. Six inoculated or mock-inoculated kernels in glass vials were kept at room temperature for 3.5 and 7 days, respectively. Upon harvesting, the kernels were immediately frozen in liquid nitrogen and ground to fine powder and were lyophilized and used for measurement of oxylipins and fatty acids.

Field evaluation of resistance to AF contamination.

Field experiments were conducted over the course of two years at two Texas Agricultural Experiment Stations: Weslaco, where the soil is a Harlingen clay, and College Station (Burlington County), where the soil is a Miller-Norwood-Pledger clay loam. Corn was planted at Weslaco on March 17, 2005 and Feb. 15, 2006 and at College Station on April 7, 2005 and March 30, 2006. Each replicate consisted of a single 7.3-m row, with 76-cm row spacing and 30-cm plant spacing, and an average of 20 plants per row. There were four replicates per genotype arranged in a randomized complete block design.

Primary ears were inoculated 10 days after mean silking date with 3 ml of an aqueous suspension containing 3×10^7 conidia of *A. flavus* NRRL 3357 (Wicklowsky et al. 1998; Windham et al. 2003), using the nonwounding silk-channel method (Zummo and Scott 1992). The conidial inoculum was prepared as described previously (Betrán and Isakeit 2004). Half of the plants within each replicate were inoculated and the rest remained uninoculated.

Ears were hand-harvested when kernel moisture was below 15%. Ears were husked, and ear rot was assessed by quantifying the proportion of kernels with visible fungal colonization (expressed as percent of infected kernels). For AF quantification, ears were shelled, and kernels were bulked and then ground using a Romer mill (Romer Labs, Union, MO, U.S.A.). AF was quantified from 50-g subsamples using the Vicam AflaTest fluorometer (Vicam), according to the USDA FGIS protocol as described above.

Statistical analysis.

Kernel assays. For conidial production in kernel assays, sample means were compared using analysis of variance (ANOVA) ($\alpha = 0.05$, Tukey-Kramer means comparisons). For oxylipins and AF concentrations from kernel assays, means were compared via ANOVA ($\alpha = 0.05$), and Fisher's protected least significant difference (LSD) test was used to separate means. For ST concentrations from kernel assays, differences between infected WT and *lox3-4* mutant kernels were assessed using Student's *t* test.

Field samples. AF concentrations, measured in ng/g, were log-transformed to equalize variance. Means were compared via ANOVA ($\alpha = 0.05$) and Fisher's protected LSD test was used to separate means.

Statistical tests were done using the SPSS statistical package (SPSS Inc., Chicago) or JMP, version 3 (SAS Institute, Cary, NC, U.S.A.).

Distribution of materials.

Novel materials described in this publication may be available for noncommercial research purposes upon acceptance and signing of a material transfer agreement. In some cases such materials may contain or be derived from materials obtained from a third party. In such cases, distribution of material

will be subject to the requisite permission from any third-party owners, licensors, or controllers of all or parts of the material. Obtaining any permission will be the sole responsibility of the requestor. Plant germplasm will not be made available except at the discretion of the owner and then only in accordance with all applicable governmental regulations.

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