Oxygenase Coordination Is Required for Morphological Transition and the Host–Fungus Interaction of *Aspergillus flavus*

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Submitted 10 November 2008. Accepted 28 March 2009.

Oxylipins, a class of oxygenase-derived unsaturated fatty acids, are important signal molecules in many biological systems. Recent characterization of an *Aspergillus flavus* lipoyxygenase gene, *lox*, revealed its importance in maintaining a density-dependent morphology switch from sclerotia to conidia as population density increased. Here, we present evidence for the involvement of four more oxylipin-generating dioxygenases (*PpoA*, *PpoB*, *PpoC*, and *PpoD*) in *A. flavus* density-dependent phenomena and the effects of loss of these genes on aflatoxin production and seed colonization. Although several single mutants showed alterations in the sclerotia-to-conidia switch, the major effect was observed in a strain downregulated for all five oxygenases (invert repeat transgene [IRT] strain IRT4 = *ppoA*, *ppoB*, *ppoC*, *ppoD*, and *lox*). In strain IRT4, sclerotia production was increased up to 500-fold whereas conidiation was decreased down to 100-fold and the strain was unable to switch into conidial production. Aflatoxin (AF) production for all mutant strains and the wild type was greatest at low population densities and absent in high populations except for strain IRT4, which consistently produced high levels of the mycotoxin. Growth on host seed by both IRT4 and IRT2 (downregulated in *ppoA*, *ppoB*, and *ppoD*) was marked by decreased conidial but increased AF production. We propose that *A. flavus* oxygenases and the oxylipins they produce act in a highly interdependent network with some redundancy of biological function. These studies provide substantial evidence for oxylipin-based mechanisms in governing fungus–seed interactions and in regulating a coordinated quorum-sensing mechanism in *A. flavus*.

Aspergillus flavus is an opportunistic pathogen of crops. It is important because it produces aflatoxin (AF) as a secondary metabolite in the seed of a number of crops both before and

after harvest. AF is a potent carcinogen that is highly regulated in most countries (Bhatnagar et al. 2006; Klich 2007; Robens and Cardwell 2003). *A. flavus* generates asexual spores (conidia) which serve as the primary disseminating and infectious unit. *A. flavus* also forms resting structures called sclerotia, hardened, pigmented mycelial tissues capable of long periods of dormancy during unfavorable growth conditions (Diener et al. 1987). Sclerotia-infected plant tissue such as corn kernels, cobs, and leaf tissue can remain in the soil and support the fungus until the following season when newly exposed mycelium or sclerotia can give rise to conidial structures, thus producing the primary inoculum for the next infection cycle (Diener et al. 1987; Hedayati et al. 2007; Scheidegger and Payne 2003).

Understanding how *Aspergilli* perceive environmental cues and adjust their morphological strategies is crucial for understanding how to address the agricultural and health damage they cause. Several known stimuli affect *Aspergillus* spp. development and reproduction, including temperature, pH, carbon and nitrogen sources (Bennett et al. 1979; Cotty 1988; Gqaleni et al. 1997; Keller et al. 1997), air availability (Guzman-de-Peña and Ruiz-Herrera 1997), and the circadian oscillator (Greene et al. 2003). In addition, exogenous (seed-derived) and endogenous (*Aspergillus*-derived) signaling compounds called oxylipins also affect development in the *Aspergilli* (Calvo et al. 1999; Tsitsigiannis and Keller 2007; Tsitsigiannis et al. 2004).

Oxylipins are a broad class of oxygenated polyunsaturated fatty acids that function as molecular signals in plants, animals, insects, and fungi (Funk 2001). In fungi, oxylipins have emerged as key signals conserved among ecologically diverse groups. Oxylipins affect sporulation in *Neurospora*, *Alternaria*, and *Aspergillus* spp. (Champe et al. 1987; Hyeon 1976; Nukima et al. 1981) and toxin biosynthesis in *Aspergillus* spp. (Calvo et al. 2002; Tsitsigiannis and Keller 2006) and *Fusarium* spp. (McDonald et al. 2004); and act as pathogenicity-related signals for quorum sensing and phase transition in yeast and dimorphic fungi (Shea et al. 2006; Tsitsigiannis and Keller 2007). Corroborating these findings, recently it has been shown that *A. flavus* possesses a quorum-sensing-like mechanism, where a sclerotia-to-conidia transition is governed by cell density and lipoyxygenase activity (Horowitz et al. 2008).

In plants, the enzymes responsible for forming oxylipins from polyunsaturated fatty acids are called lipoyxygenases (LOX), which incorporate molecular oxygen into fatty acids at either the 9- or 13-carbon position (9-LOX and 13-LOX, respectively) (Liavonchanka and Feussner 2006; Nemchenko et al. 2006). In fungi, a family of lipid dioxygenases known as Ppo

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Nucleotide sequences data reported here are available in the GenBank database under the accession numbers FJ263387 for *ppoA*; FJ263388 for *ppoB*; FJ263389 for *ppoC*; FJ263390 for *ppoD*.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplemental figures are published online. In addition, Figure 6 is in color online.

oxygenases are conserved in many filamentous fungi and produce oxylipins that are structurally and physiologically similar to those produced by plant lipoxygenases (Champe and El-Zayat 1989; Champe et al. 1987; Hyeon 1976; Nukima et al. 1981; Tsitsigiannis and Keller 2007). Several lines of evidence suggest that plant and fungal oxylipins can functionally substitute for each other and are involved in fungus–host communications. Exogenously applied plant 9-LOX and 13-LOX linoleic acid-derived oxylipins have sporogenic capabilities comparable with the fungal-derived Ppo oxylipins (Calvo et al. 1999). Furthermore, 9-LOX-derived oxylipins increase mycotoxin biosynthesis, including AF, whereas 13-LOX-derived oxylipins inhibit biosynthesis (Burow et al. 1997), phenotypes reminiscent of specific *ppo* mutations in *A. nidulans* (Tsitsigiannis et al. 2006). Reciprocal “cross talk” between fungus and seed was also demonstrated when plant *LOX* expression was altered in response to fungal infection in both peanut and maize seed (Burow et al. 2000; Tsitsigiannis et al. 2005b, 2006) and, furthermore, when peanut *LOX* expression was muted when seed were infected by *Aspergillus* oxylipin mutants (Brodhagen et al. 2008). The response of *Aspergillus* spp. to seed oxylipins led to the postulation that host oxylipins can mimic or interfere with endogenous fungal oxylipins on a cellular basis, thus affecting fungal development and the outcome of the host–fungus interaction (Burow et al. 1997; Calvo et al. 1999). This was strengthened by experimental data when it was shown that expression of a maize 9-*LOX* gene in an *A. nidulans* Ppo mutant could partially remediate the *Appo* phenotype (Brodhagen et al. 2008) and that a maize 9-*LOX* mutant was more susceptible to *Aspergillus* spp. infections (Gao et al. 2009).

Three Ppo dioxygenases have been characterized in the model organism *A. nidulans*. Deletion of *ppoA* or *ppoB* increases the ratio of asexual to sexual spores (Tsitsigiannis et al. 2004, 2005b) while deletion of *ppoC* has the opposite phenotype—an increase in sexual spores and decrease in asexual spores (Tsitsigiannis et al. 2005b). In the human pathogen *A. fumigatus*, there was no measurable phenotype in the *ppoA* and *ppoB* mutants but the *ppoC* mutant showed several abnormalities, including decreased spore production, increased conidial size, and altered host macrophage interactions (Dagenais et al. 2008). Considering the importance of Ppos in *A. nidulans* and *A. fumigatus* biology and Lox in *A. flavus* cell density morphology transitions (Horowitz et al. 2008), we set out to determine whether the four *A. flavus* ppos (*ppoA*, *ppoB*, *ppoC*, and *ppoD*) were important in fungal development and pathogenicity. An examination of mutants containing a single *ppo* deletion as well as strains downregulated in expression of multiple *ppo* genes combined with *lox* loss, enabled assessment of the relative contribution of each gene to spore, sclerotial, and AF production as well as host colonization. The most striking phenotype was the strain deficient in all five oxygenases which resulted in blockage of the sclerotia-to-conidia transition and highly upregulated AF production. We propose that *A. flavus* Ppos and the oxylipins they produce participate in a quorum-directed developmental shift and act in a redundant but highly interdependent network that affects differentiation processes and the outcome of the host–fungus interaction.

RESULTS

A. flavus genome contains four *ppo* fatty acid peroxidases.

Four Ppo homologs of *A. nidulans* PpoA were identified in *A. flavus* genomic scaffolds. PpoA and PpoC are on chromosome 2 and PpoB and PpoD are found on chromosomes 8 and 4, respectively. Each Ppo contains a heme peroxidase domain as determined by the National Center for Biotechnology Information conserved domain search. Biology Workbench software

was used to align *A. flavus* Ppo proteins based on predicted amino acid sequence identity with Ppos from *A. nidulans* and *A. fumigatus*. Percent sequence identities are shown in supplementary data (Supplementary Fig. 1). The PpoA and PpoC groups were highly conserved and contained homologs from each of the three species; the PpoB and PpoD groups were more loosely related, although strong pairing was seen between *A. flavus* and *A. fumigatus*.

The *ppo* genes are expressed during vegetative growth and reproduction.

Because both *A. nidulans* and *A. fumigatus* *ppo* genes have been associated with sporulation processes, we examined expression of each *A. flavus* gene during conidiophore formation (Fig. 1). Only *ppoA* is expressed prior to conidiophore initiation. Its expression fluxgate during conidiophore formation but remains present from the beginning of vesicle formation and through the point when mature conidiophores appear. *ppoC* expression is detectable when the vesicles begin to produce metulae and gradually increases as mature conidia are produced. *ppoD* becomes detectable as the first conidiophores mature and, finally, *ppoB* is expressed when the bulk of conidiophores are bearing mature conidia (Fig. 1A). Relative expression of each *ppo* gene during conidiophore formation is presented in Figure 1B.

Identification and characterization of oxygenase deletion mutants and RNAi-deficient mutants.

To assess the functions of *ppo* genes in *A. flavus*, *ppoA*, *ppoC*, and *ppoD* were inactivated separately by homologous recombination with the plasmids pJBS3.1, pRAW2.2, and pJBS4.1 in the *pyrG*[−] strain NRRL 3357.5 (despite several tries, we were unable to delete *ppoB*). Southern analysis of transformants showed the replacement of each gene with *A. parasiticus* *pyrG* (Fig. 2A through C). Initially, three transformants of each genotype were examined and, when preliminary physiology experiments showed similar phenotypes, one transformant of each genotype was selected for further tests: TJBS3.1.19 for the *AppoA* genotype, TRAW2.2.15 for *AppoC*, and TJBS4.1.85 for *AppoD*. These three strains were compared with TSHB2.39 (Δ *lox* mutant) (Horowitz et al. 2008) and a wild-type strain (NRRL 3357). To create strains with multiple oxygenase lesions, RNAi technology was employed using inverted repeat transgene of several oxygenases, as depicted in Supplementary Figure 2. Strain TJBS 5.1.60 carried the invert repeat transgene (IRT) of *ppoA*, and *ppoB* in the *AppoD* background was named IRT2 (Fig. 2D). Silencing of *ppoC* and *lox* genes in IRT2 created IRT4 (*AppoD*; *ppoA*, *-B*, and *-C*; and *lox* IRT), deficient in expression of all five oxygenases (Fig. 2E). As expected for IRT-containing strains, transcripts (*ppoA* and *ppoB* in IRT2 [Fig. 2D] and *ppoA*, *ppoC*, and *lox* in IRT4 [Fig. 2E]) were degraded in these strains.

Conidial and sclerotial density-dependent development is impaired in oxygenase mutants.

Based on recent data where deletion of *lox* in *A. flavus* affected the sclerotia-to-conidial population shift (Horowitz et al. 2008), we predicted that *ppo* mutants would exhibit an altered density-dependent phenotype. The relative ability of the wild type, *AppoA*, *AppoC*, Δ *lox*, *AppoD*, IRT2, and IRT4 to form sclerotia and conidia was determined by inoculating 10², 10⁴, and 10⁶ conidia on glucose minimal medium (GMM) plus 2% sorbitol plates which were placed in constant darkness at 29°C for 7 days. Cell density development patterns were skewed to various degrees among the oxygenase mutants compared with the wild type (Supplementary Fig. 3). Macroscopically, *AppoC*, Δ *lox*, IRT2, and IRT4 showed alterations in the

sclerotia-to-conidia shift. In particular, IRT4 exhibited the most striking phenotype, with increased production of sclerotia and near absence of conidia at all populations. Conidia production decreased in $\Delta ppoC$, Δlox , IRT2, and IRT4 strains at all population densities. IRT4 showed the most dramatic decrease in conidiation: 75, 86, and 97% reduction compared with the wild type at 10^2 , 10^4 , and 10^6 conidia/plate, respectively. In contrast to these mutants, the $\Delta ppoD$ strain showed an increase in conidiation compared with the wild type at 10^6 conidia/plate (Fig. 3A). At low cell density (10^2 conidia/plate), there was no difference in sclerotial production as measured by weight between any strains (Fig. 3B). At the intermediate population level (10^4 conidia/plate), $\Delta ppoD$ showed a reduction in sclerotial production compared with all other mutants (Fig. 3B). At high cell densities, all strains containing a down-regulated or deleted *ppoC* or *lox* allele exhibited high sclerotial production. The loss of *ppoA* showed a minimal effect, with slightly more sclerotia formation at the highest cell density. In contrast, $\Delta ppoD$ was the only strain to produce less sclerotia than the wild type, which correlated with its increased conidial production (Fig. 3A and B). The most striking phenotype was the greatly increased production of sclerotia in IRT4 compared with the wild type by 114 and 532% at 10^4 and 10^6 cell densities, respectively (Fig. 3B).

Genes *ppoC* and *lox* cooperate in a synergistic manner to regulate cell density development.

The IRT2 strain (e.g., *ppoA*, *ppoB*, and *ppoD* downregulated) did not show as much sclerotial increase as the IRT4 strain; therefore, it appeared that *PpoC* or *Lox* was most critical the overproduction of sclerotia by IRT4 (Fig. 3B). To assess the contribution of *ppoC* and *lox* individually to the IRT4 phenotype, *ppoC* and *lox* were next inactivated independently in the IRT2 background to yield IRT2- Δlox and IRT2- $\Delta ppoC$. Sclerotia and conidial production were examined in 6-day-old cultures (Fig. 4A and B). Both the IRT2- Δlox and IRT2- $\Delta ppoC$ strains produced an identical phenotype, intermediate to that of IRT2 and IRT4 (Fig. 4A).

Secondary metabolite production, including AF, is regulated by cell density and disturbed in oxygenase mutants.

To evaluate the effect of cell density on secondary metabolite biosynthesis, including AF (B1), cultures of *A. flavus* wild type at 10^2 to 10^7 conidia per plate were grown on GMM (to promote general secondary metabolite biosynthesis) (Fig. 5A, upper panel) or GMM plus 2% sorbitol (to promote AF biosynthesis) (Fig. 5A, lower panel) in constant darkness at 29°C for 6 and 3 days, respectively. Thin-layer chromatography (TLC) analysis

Fig. 1. Expression of *ppo* during conidiophore formation. **A**, *Aspergillus flavus* wild-type 3357 grown in liquid shaking culture (270 rpm, 29°C) to competence (22 h) and transferred to filter paper on 2% sorbitol solid glucose minimal medium at 29°C under constant light. Total RNA was extracted from three replicates at 0, 6, 12, 24, and 48 h after the transfer. Northern blots were probed with internal fragments of each *ppo*. Below the Northern blots is a cartoon depiction of the predominant fungal tissue at each time point—competent tissue, vesicle, vesicle covered with phialides, young conidiophores, and mature conidiophores at each time point, respectively. Ethidium bromide-stained rRNA is shown as a loading control. **B**, Quantification of band intensity was performed by Image J software and expressed in terms of relative intensity compared with the standard. Statistical significance of quantification was assessed using Tukey-Kramer multiple comparison test ($P < 0.05$).

showed that secondary metabolite production is density dependent in the wild type, where increasing cell density yields the lowest secondary metabolite production (Fig. 5A)—a pattern similar to that of sclerotial production. AF was detected only at the lowest cell density (10^2 conidia/plate) under the experimental conditions assayed (Fig. 5A, lower panel).

To study the role of oxygenases specifically on AF biosynthesis, we examined AF production in all mutants grown on GMM plus 2% sorbitol at three cell densities: 10^2 , 10^4 , and 10^6 (Fig. 5B). AF was detected in all strains at the lowest cell density. There was minimal AF production in $\Delta ppoC$, Δlox , and IRT2 strains at the intermediate concentration of 10^4 conidia/plate;

however, the most striking finding was the high level of AF production in IRT4 at all population densities (Fig. 5B). To address the impact of *ppoC* and *lox* individually on AF production in the IRT4 strain, we examined the IRT2- $\Delta ppoC$ and IRT2- Δlox strains for their ability to produce AF at high cell densities. The results demonstrate that reduction of both *ppoC* and *lox* is required to induce AF at high density (Fig. 5C).

IRT2 and IRT4 mutants are impaired in conidiation but not AF production on peanut and corn seed.

To dissect the role of *A. flavus* oxygenases when the fungus is interacting with live seed, we examined the ability of

Fig. 2. Deletion and/or silencing expression of *ppo* genes in *Aspergillus flavus*. **A**, Diagram of the *A. ppoA*; **B**, *ppoC*; and **C**, *ppoD* coding sequence and how they were replaced with the *A. parasiticus pyrG* selectable marker by homologous recombination to generate the single deletion $\Delta ppoA$, $\Delta ppoC$, and $\Delta ppoD$ mutants, respectively. Southern analysis of wild-type NRRL 3357 strain (WT), $\Delta ppoA$ mutant TJBS3.1.19, $\Delta ppoC$ mutant TRAW2.2.15, and $\Delta ppoD$ TJBS4.1.85. To determine deletion of *ppoA*, *ppoC*, and *ppoD*, genomic DNAs were digested with *NcoI*, *BamHI*, *HindIII*, *BamHI*, and *Sall* as shown. *PpoA*: Expected hybridization band patterns by *NcoI* digestion are a 0.5- and 1.0-kb band for the WT strain and a 4.7-kb band for $\Delta ppoA$ strains (using the 3' *ppoA* flank as a probe). When the 5' flank was used as a probe, the expected hybridization band patterns by *NcoI* digestion are 5.5 kb for the WT strain and 4.1 kb for $\Delta ppoA$ mutants. *PpoC*: Expected hybridization band patterns for *BamHI* are 2- and 5.5-kb bands for the WT strain and 1.5- and 8-kb bands for $\Delta ppoC$ strains. Digestion with *HindIII* is a 2-kb band for the WT and a 4-kb for $\Delta ppoC$ when full-length *ppoC* is used as a probe. *PpoD*: Expected hybridization band patterns with *BamHI* is a 2.5-kb band for the WT strain and a 4.7-kb for $\Delta ppoD$ strains with the 5' *ppoD* flank as a probe. Digestion with *HindIII* gives a 5.3-kb band for the WT and ≥ 7 kb for $\Delta ppoD$ with the 3' *ppoD* flank as a probe. **D**, Northern blots analysis to confirm if RNAi machinery successfully silenced both *ppoA* and *ppoB* genes in a $\Delta ppoD$ background is demonstrated. Expression of *ppo* in TJBS 5.1.60 (invert repeat transgene [IRT] strain IRT2) and the WT (NRRL 3357). In all, 10^6 conidia from 5-day-old cultures were grown in liquid shaking culture (270 rpm, 29°C) to competence (22 h) and transferred to filter paper on 2% sorbitol solid glucose minimal medium (GMM) at 29°C under constant light. Total RNA was extracted from two replicates at 0, 24, and 48 h after the transfer. The presence of a smear for *ppoA* and *ppoB* in the IRT2 strain indicated a degraded transcript of the silenced genes as opposed to full-length transcripts in the WT. **E**, Northern blot analysis to study how *lox*, *ppoA*, and *ppoC* are silenced in IRT4 mutant. WT *A. flavus*, IRT2 (TJBS 5.1.60), and IRT4 (TSHB5.57) mutants were grown in liquid shaking culture (270 rpm, 29°C) to competence (22 h) and transferred to 2% sorbitol GMM liquid medium at 29°C under constant light, and total RNA was extracted from two replicates 2 h after the transfer. The presence of a smear for *ppoA* (control), *ppoC*, and *lox* indicated a degraded transcript of the silenced genes as opposed to full-length transcripts in the WT. Actin probe was used as a control to demonstrate equal RNA loading.

A. flavus oxygenase mutants to conidiate and produce AF in peanut (Fig. 6) and corn (Fig. 7) seed. IRT2 and IRT4 strains consistently produced less conidia than the wild type on both peanut and corn seed (Figs. 6 and 7). Conidial production was decreased 85% for both IRT2 and IRT4 compared with the wild type on peanut ($P \leq 0.05$) and 90 to 97% on corn. However, despite the low levels of conidiation on the seed, IRT2 and IRT4 produced the most AF in the seed (Figs. 6B and 7B).

Fig. 3. Conidiation levels and sclerotia production are affected by oxygenase mutants. In all, 10^2 , 10^4 , and 10^6 conidia of wild-type strain NRRL 3357 (WT) and $\Delta ppoA$, $\Delta ppoC$, $\Delta ppoD$, Δlox , invert repeat transgene (IRT)2, and IRT4 were overlaid on 10 ml of glucose minimal medium plus 2% sorbitol and placed in constant darkness at 29°C for 3 days for assessment of conidiation levels and 7 days for assessment of sclerotia production. **A**, Conidial production was determined by counting under the microscope. Values are means of four replicates and tests were repeated three times in order to examine reproducibility. Spore suspensions were freshly prepared for each test. Letters (a, b, c, and d) indicate statistical differences ($P < 0.05$) at different cell densities according to Tukey-Kramer multiple comparison test. **B**, Sclerotia production was determined gravimetrically (dry weight as milligrams per plate). Values are means of four replicates and tests were repeated three times in order to examine reproducibility. Spore suspensions were freshly prepared for each strain. Distances larger than the vertical bars between symbols at each cell density are significant ($P \leq 0.05$) according to Tukey-Kramer multiple comparison test.

DISCUSSION

Density-dependent development in *A. flavus* is regulated through oxygenase expression.

In this study, we have characterized *A. flavus* fatty acid mutants deleted or downregulated for five oxygenase genes. Our findings provide evidence supporting an oxygenase signaling network that affects multiple developmental processes, including a quorum-like morphological shift from sclerotia to conidia formation, secondary metabolism, and host seed colonization. Our results further suggest that a pool of selected oxylipins are important for density-dependent morphology shifts in this fungus. The phenotypes of the individual oxygenase mutants in *A. flavus* suggested a possible redundancy in function. To address this possibility, two strains with multiple lesions were created. IRT2, a strain in which *ppoA* and *ppoB* were suppressed by RNAi in the $\Delta ppoD$ background, was reduced by almost 50% in conidiation compared with the wild type. Downregulation of all five oxygenases in *A. flavus* strain IRT4 resulted in overproduction of sclerotia and minimal conidiation through all cell densities. These results support the hypothesis that single *ppo* deletion phenotypes may be masked or compensated by activity of other Ppos.

Conserved oxylipin signaling among Aspergilli.

In *A. nidulans*, independent inactivation of the three *ppo* genes resulted in alterations in the ratios of asexual to sexual spores produced. $\Delta ppoA$ and $\Delta ppoB$ strains were both increased in this ratio, whereas $\Delta ppoC$ strains displayed the opposite phenotype—increased in sexual spore and decreased in asexual spore production (Tsitsigiannis et al. 2004; 2005b). In *A. fumigatus*, the *ppoC* mutant showed several abnormalities, including a decrease in spore production, aberrant conidiophore formation, and altered cell wall morphology (Dagenais et al. 2008; Garscha et al. 2007; Tsitsigiannis et al. 2005a). Although *A. flavus ppoD* deletion showed an increase in asexual reproduction (similar to *A. nidulans ppoA* and *ppoB* mutants), the $\Delta ppoC$ and Δlox strains independently displayed a significant decrease in conidiation compared with the wild type at the high cell density (Fig. 3). Inactivation of PpoC—a highly conserved protein—shows some similarity among the three Aspergilli. In *A. nidulans*, $\Delta ppoC$ overproduces its resting stage, the cleistothecium, concomitant with decreased conidial production (Tsitsigiannis et al. 2004). In the asexual fungus *A. fumigatus*, $\Delta ppoC$ mutants also displayed decrease in conidiation (Dagenais et al. 2008). Results here show that *A. flavus ppoC* mutants share a similar decrease in conidial production accompanied with overproduction of sclerotia at the high cell densities. This phenotype is very similar to deletion of the *A. flavus lox* gene (Fig. 3) (Horowitz et al. 2008). The PpoC protein has recently been found to produce 10R-hydroxy-8E, 12Z-octadecadienoic acid as its major oxylipin (Garscha et al. 2007). Possibly, this is a significant signaling molecule in *Aspergillus* spp. conidial development. A summary of the mutant phenotypes among the three Aspergilli species (Table 1) demonstrates some conserved oxylipin signaling patterns governing development.

AF production is modulated by oxygenase activity.

Lack of all three *ppo* genes in *A. nidulans* resulted in a strain unable to produce the mycotoxin sterigmatocystin (a precursor to AF) but upregulated in penicillin production (Tsitsigiannis et al. 2006). Here, we observed that, in the *A. flavus* IRT4 strain (reduced in expression of all five oxygenases), there was a dramatic increase in AF through all the cell densities (Fig. 5B). Our findings demonstrate that a combination of oxygenase activity is required for AF regulation.

Oxylipin-mediated host pathogen cross talk.

Recent studies have uncovered a complex fungus–host interaction governed by oxylipins (Brodhagen et al. 2008; Gao et al. 2009). Here, we assessed the effect of deletion of oxylipin biosynthesis genes in *A. flavus* on disease seed colonization. Both IRT2 and IRT4 were significantly reduced in conidiation on peanut and corn seed compared with the wild type, yet contaminated the seed with high levels of AF (Figs. 6 and 7). These findings suggest that naturally occurring differences in oxygenase activity could explain some differences in AF production in field populations of *A. flavus* (Horn 2007) and also support reports where high levels of AF were found despite little evidence for fungal infection (Cotty and Mellon 2006).

Interestingly, conidiation and toxin production by *A. flavus* were increased during colonization of a maize *lox3-4* mutant, as recently reported by Gao and associates (2009). Together with the data reported here, these observations suggest that at least certain oxylipins, directly or indirectly, act to limit AF

production in situ. This interaction of fungal pathogen and the maize host is pathogen specific: in contrast to infection by *Aspergillus* spp., the maize *lox3-4* mutant was more resistant to *Fusarium verticillioides* and *Colletotrichum graminicola* infections (Gao et al. 2007). The latter results may reflect many synergistic, opposing, or compensatory interactions among the fungal and seed oxylipin pool and serve to illustrate that potential oxylipin-based control strategies may need to be specified for each fungus–host interaction.

Oxygenases as population-density-dependent determinants of fungal development.

Quorum sensing is a density-dependent process that functions when a population is of certain size. Development and survival of any organism depends in large part on proper responses to the density of the surrounding population. In recent years, a quorum-sensing-like phenomena was uncovered in several yeast species. Each of these cases involves a morphological transition among dimorphic fungi, from a filamentous my-

Fig. 4. *ppoC* and *lox* cooperate in a synergistic manner to regulate cell density development. **A**, To assess the contribution of *ppoC* and *lox* individually to the invert repeat transgene (IRT)4 phenotype, wild-type strain NRRL 3357 (WT), single deletion of Δlox and $\Delta ppoC$, IRT2- $\Delta ppoC$, and IRT2- Δlox were compared with IRT2 and IRT4 strains. In all, 10^6 conidia were overlaid on 10 ml of glucose minimal medium plus 2% sorbitol and placed in constant darkness at 29°C for 6 days. Sclerotia production was determined gravimetrically (dry weight as milligrams per plate) and conidia by counting under the microscope. Values are means of four replicates and tests were repeated three times in order to examine reproducibility. Spore suspensions were freshly prepared for each test. Different letters (a, b, and c for conidia and A, B, and C for sclerotia) indicate statistical differences ($P < 0.05$) according to the Tukey-Kramer multiple comparison test. Data counted only for the high cell density, 10^6 conidia/ml. **B**, Visual phenotype of sclerotia production at 10^6 conidia/plate after 6 days of incubation; the additive effect of *ppoC* and *lox* deletion is demonstrated by increased sclerotia production in IRT4 compared with independent single deletion of *ppoC* and *lox* in IRT2 background.

celial form to a yeast form, or vice versa (Sprague and Winans 2006). Investigations of quorum sensing in *Candida albicans* identified two quorum-sensing molecules: farnesol, which inhibits formation of germ tubes when cultures reach high density, and tyrosol, which promotes cell growth and the formation of germ tubes at low cell density (Chen and Fink 2006; Chen et al. 2004). Whole-genome microarray expression analysis suggest that these *C. albicans* quorum-sensing molecules may also play a role in signaling the transition to the stationary phase (Chen and Fink 2006). Recently, we reported that *A. flavus* not only demonstrates a quorum-sensing mechanism as illustrated in the sclerotia-to-conidia switch but also that Lox activity is important for this switch (Horowitz et al. 2008). Here, we show that the four Ppo proteins also play a critical role in this switch and that oxygenase activity also impacts an apparent density-dependent regulation of secondary metabolism, possibly related to sclerotial formation. It appears that the activity of PpoC and Lox oppose the role of PpoD in *A. flavus* development (Table 1), much like that seen in PpoC versus PpoB in *A. nidulans*. The accumulating data suggest that control of phase change and differentiation in *A. flavus* is modulated in part by oxylipins.

Quorum sensing can provide a means to respond effectively to changes in environmental opportunities and allow the fungus to respond to nutrient availability and microbial competition. In *A. flavus*, a sentinel system regulating conidial and sclerotial shifts could be evolutionarily beneficial for the organism to help it respond to changing environmental conditions. The conidium is the dispersal spore expected to be produced at times of starvation, a condition that may arise as high populations compete for limited nutrients. Sclerotial production would be favorable in establishing a niche for the producing organism and deterring predators through production of toxic metabolites (Wicklow et al. 1982).

MATERIALS AND METHODS

Fungal strains and growth conditions.

A. flavus strains used and generated in this study are listed in Table 2. All strains used for physiological studies were prototrophic. All strains were grown at 29°C and maintained on GMM (Shimizu and Keller 2001) unless otherwise indicated. Cells were visualized by using an Olympus BX60F-3 microscope and an Olympus SZ-60 stereoscope.

Assay for conidia and sclerotia density dependence.

Conidial and sclerotial measurements followed the procedure outlined by Horowitz and associates (2008). Briefly, 10 ml of 1.6% GMM + 2% sorbitol agar was overlaid with 3 ml of 0.7% GMM agar + 2% sorbitol agar containing 10^2 (low cell density), 10^4 (intermediate cell density), or 10^6 (high cell density) conidia/plate of each appropriate strain in a 60-by-15-mm petri plate. Plates were incubated at 29°C under continuous darkness for 7 days. To accurately visualize the sclerotia, plates were sprayed thoroughly with 70% ethanol in water to remove conidia and aerial mycelia. The exposed sclerotia then were collected and lyophilized, and sclerotium production was determined gravimetrically (dry weight per plate). Conidia were counted at 3 or 6 days. To accurately count conidia, three 1-cm plugs were homogenized in 3 ml of 0.01% Tween 80 water, diluted to 1× and counted with a hemacytometer at ×400 magnification. For each treatment, conidia and sclerotia counts were performed using four replicates and tests were repeated three times in order to examine reproducibility. Spore suspensions were freshly prepared for each test.

Secondary metabolites analysis.

For total secondary metabolites analysis, 10^2 to 10^7 conidia per plate of the wild type were inoculated at each power of 10

Fig. 5. Secondary metabolites and aflatoxin (AF) density dependence is affected by *Aspergillus flavus* oxygenases. **A**, Cell density regulates general secondary metabolites, including AF in *A. flavus*. In all, 10^2 – 10^6 conidia of wild-type strain NRRL 3357 (WT) were overlaid stepwise to each power of 10 on 10 ml of glucose minimal medium (GMM) alone for general secondary metabolites or plus 2% sorbitol for AF and placed in constant darkness at 29°C for 7 or 3 days, respectively. Metabolites were extracted from three 1-cm plugs in 1 ml of chloroform and separated by thin-layer chromatography (TLC). **B**, To assess AF production in *A. flavus* oxygenase mutants, 10^2 , 10^4 , and 10^6 conidia from 3-day-old cultures were overlaid on 10 ml of GMM plus 2% sorbitol in constant darkness. Silencing the expression of four *A. flavus* ppos and *lox* genes in $\Delta ppoD$ led to increased levels of AF production through all cell densities. **C**, Relative individual effect of *ppoC* and *lox* deletion on AF production in invert repeat transgene (IRT)2 background. Reduction of both *ppoC* and *lox* genes is required to induce AF at high cell densities, demonstrating the synergistic effect of both *ppoC* and *lox* on AF production observed in IRT4 mutant. TLC analyses were repeated three times and quantified by image J software. Quantification of band intensity was expressed in terms of relative intensity compared with AF standard within the same TLC plate. Statistical significance of quantification was assessed using Tukey-Kramer multiple comparison test ($P < 0.05$).

on GMM and placed in constant darkness at 29°C for 7 days. For AF analysis, GMM + 2% sorbitol medium was overlaid with 3 ml of a suspension of appropriate strains at 10^2 , 10^4 , or 10^6 conidia/ml that were grown for 3 days at 29°C. Three 1.5-cm-diameter cores were harvested from the center of each plate and homogenized in 5 ml of distilled water. Chloroform (1 ml) was added and the mixture was vortexed for 1 min, then centrifuged at 2000 rpm for 10 min. The lower organic layer was removed and evaporated. Residue was resuspended in 100 μ l of chloroform and 10 μ l of the suspension were spotted onto silica gel TLC plates (Whatman Ltd., Ken, England). Extracts were separated on the TLC plates using a benzene/methanol/acetic acid (80:15:5, vol/vol/vol) solvent system. Experiments were done in triplicate and repeated twice in order to examine reproducibility. AF standard was purchased from Sigma-Aldrich (St. Louis). AF was visualized using long-wave (366 nm) UV light and digital photographs were taken. Quantification of AF band intensity was performed by Image J software (version 1.24; developed at the National Institutes of Health) and expressed in terms of relative intensity compared with the AF standard. Statistical significance of quantification was assessed by mean comparisons according to the Tukey-Kramer multiple comparison test at $P \leq 0.05$. Mean values with different letters are significant.

Peanut and corn seed infection studies and AF analysis from seed.

Infection studies and AF analysis of peanut (*Arachis hypogaea*) cv. SunRunner and untreated maize seed (X516XW raw)

obtained from Kaltenberg Seed Farm, Inc. (Waunakee, WI, U.S.A.) were performed as has been described previously (Burow et al. 2000; Tsitsigiannis and Keller 2006). For peanut infection, mature peanut seed were prepared by removing the brown exterior peanut layer (testa). The two cotyledons of each seed were separated and the embryo carefully removed without damaging any of the cotyledons tissue. Then, cotyledons were surface sterilized by placing them in a tea ball infuser and dipping them in a beaker containing 0.05% sodium hypochlorite in sterile water for 3 min. The tea ball was transferred to a new beaker containing sterile distilled water for 30 s, followed by a 5-s wash with 70% ethanol in a new beaker and one more 30-s wash with sterile distilled water while shaking the tea ball. The cotyledons were drained completely and placed in a petri dish until the time of infection. All steps were aseptically performed in a biosafety hood. Peanut cotyledons were inoculated with a 10^5 spore/ml. Cotyledon treatments included water control (mock inoculation) and infection with fungal strains. For all treatments, 20 peanut cotyledons were immersed in 20 ml of sterile distilled water (control) or sterile distilled water with fungal conidia in 50-ml centrifuge tubes while shaking for 30 min in a rotary shaker at 500 rpm. Cotyledons were placed in petri dishes lined with three pieces of moist filter paper and a water reservoir to maintain high humidity. Cotyledons were incubated for 3 days at 29°C in dark conditions. For maize infection, untreated maize seed were inoculated similarly to peanut seed with the following differences. Seed were wounded by creating a small hole near the

Fig. 6. A and B, Growth and aflatoxin (AF) production on peanut seed. Colonization of peanut seed as reflected by conidiation levels: Wild type NRRL 3357 (WT), $\Delta ppoA$, $\Delta ppoC$, $\Delta ppoD$, Δlox , invert repeat transgene (IRT)2, and IRT4 were grown on peanut seed for 2 days at 29°C and **C**, conidia were counted from four replicates containing 20 seeds each. Columns with the same letters are not significantly different at significance level of $P < 0.05$. AF production in situ in peanut as examined by thin-layer chromatography (TLC) is shown in the lower panel. Water = mock inoculated with water and AF = AF B1 standard. TLC analyses were quantified by image J software and band intensity was expressed in terms of relative intensity compared with AF standard within the same TLC plate. Columns with the same letters are not significantly different at significant level of $P < 0.05$.

embryo and then seed were immersed in 20 ml of sterile distilled water (control) or sterile distilled water with fungal conidia at 10^5 conidia/ml in 50-ml centrifuge tubes while shaking for 30 min in a rotary shaker at 50 rpm. Ten seeds per replicate were transferred to the humidity chamber and incubated in the dark at 29°C for 3 days. The filter paper was moistened daily and any germinating seed were removed and discarded. Analysis was performed on the same number of seed for each treatment and replication. All seed experiments were repeated three to four times. For AF extraction 3 days after infection, peanut cotyledons were collected in 50-ml centrifuge tubes with the addition of 3 ml of 0.01% Tween 80 (vol/vol, water) and vortexed vigorously for 1 min. One milliliter was removed from each sample for conidia counting prior to AF extraction. For extraction, 5 ml of acetone was added to the samples followed by shaking for 10 min at 150 rpm. Samples were allowed to stand for an additional 10 min at room temperature, vortexed briefly, and centrifuged for 15 min at 2,000 rpm to collect the organic lower phase. Samples were then dried completely. The presence of abundant seed lipids in the samples hampered the clear observation of AF on TLC plates and a second extraction purification was carried out as follows. Samples were resuspended in 5 ml of 0.1 M NaCl methanol/water (55:45) and 2.5 ml of hexane and vortexed vigorously at high speed for 1 min. Samples were centrifuged at 2,000 rpm for 5 min. The hexane layer was collected and the fatty acid inter-

phase layer was discarded. The remaining aqueous phase was washed an additional time with 2.5 ml of hexane as described above. The hexane extracts were combined, allowed to dry, and then resuspended in 500 μ l of chloroform before 10 μ l of each extract was separated on a silica gel TLC plate using a chloroform/acetone (95:5, vol/vol) solvent system. Maize seed were extracted similarly but without the needed extra hexane extraction for peanut seed. Quantification of band intensity was performed by Image J software (version 1.24, developed at the National Institutes of Health) and expressed in terms of relative intensity compared with AF standard. Statistical significance of quantification was assessed by mean comparisons according to the Tukey-Kramer multiple comparison test at $P \leq 0.05$. Mean values with different letters are significant.

Nucleic acid analysis.

The extraction of DNA from fungi, restriction enzyme digestion, gel electrophoresis, blotting, hybridization, and probe preparation were performed by standard methods (Sambrook et al. 1989). To examine *ppoA*, *ppoB*, *ppoC*, and *ppoD* gene expression, wild-type NRRL 3357 was grown to competency by inoculating 500 ml of liquid GMM with 10^6 conidia/ml and shaking at 250 rpm for 22 h. The competent tissue was divided into 50-ml aliquots, filtered onto 7-cm Whatman coarse filter paper, and grown on 2% sorbitol GMM in continuous white light at 29°C for 0, 6, 12, 24, or 48 h. Total RNA was extracted

Fig. 7. Growth and aflatoxin production on corn seed. **A**, Colonization of corn seed as reflected by conidiation levels: Wild type NRRL 3357 (WT), $\Delta ppoA$, $\Delta ppoC$, $\Delta ppoD$, Δlox , invert repeat transgene (IRT)2, and IRT4 were grown on corn seed for 3 days at 29°C and conidia were counted from four replicates containing 10 seeds each. Columns with the same letters are not significantly different at significance level of $P < 0.05$. **B**, AF production in situ in corn as examined by thin-layer chromatography (TLC) is shown in the lower panel. Water = mock inoculated with water and AF = AF B1 standard. TLC analyses were quantified by image J software and band intensity was expressed in terms of relative intensity compared with AF standard within the same TLC plate. Columns with the same letters are not significantly different at significant level of $P < 0.05$.

from lyophilized mycelia using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Approximately 20 µg total RNA was run in a 1.2% agarose, 1.5% formaldehyde gel and then transferred to Hybond-XL membranes (Amersham Bioscience, Piscataway, NJ, U.S.A.) by capillary action. RNA transcript analysis was performed using random primer radiolabeled DNA fragments (Sambrook et al. 1989) derived from genomic polymerase chain reaction (PCR) products. The expression studies were performed with *ppo*-specific DNA probes which were generated from genomic DNA using the following primer combinations for each *ppo* gene: for *ppoA*, a 1.17-kb PCR fragment was amplified with *ppoA* Int Fwd and *ppoA* Int rev; for *ppoB*, a 0.85-kb PCR fragment was amplified with *ppoB* Int Fwd and *ppoB* Int rev; for *ppoC*, a 1.4-kb PCR fragment was amplified with *ppoC* Int Fwd and *ppoC* Int rev; and, for *ppoD*, a 1.3-kb PCR fragment was amplified with *ppoD* Int Fwd and *ppoD* Int rev. Primer sequences are listed in Table 3. Detection of signals was carried out with a Phosphorimager-SI (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The resulting expression analysis was measured by means of the public National Institutes of Health image processing program Image

J and are presented as relative intensity compared with time 0 presenting the vegetative growth stage. Two separate repetitions of this experiment yielded similar results. Nucleotide sequences were analyzed and compared using Sequencher (Gene Codes) and ClustalW programs (Chenna et al. 2003).

Replacement and IRT constructs of *Aspergillus flavus ppo* and *lox* genes.

The *ppo* genes were identified by nucleotide (blastn) search of the genomic scaffold of *A. flavus* based on the amino acid sequence of *A. nidulans*, *A. fumigatus*, and *A. oryzae* Ppo homologs. The *ppo* deletion constructs, including the *A. parasiticus pyrG* marker gene and the *ppo* flanking sequences, were constructed using the following methodology. Oligonucleotides used in this study (Table 3) were designed based on predicted open reading frames (ORF) and intergenic regions in these contigs. These primers were used in appropriate pairs to amplify 1.0- to 1.2-kb fragments of flanking regions by PCR using *A. flavus* genomic DNA as a template. The *ppo* flanking-region PCR products were first subcloned into PCR2.1 TOPO (Invitrogen) and then into pJW24 (Calvo et al. 2004) which

Table 1. Synopsis of oxygenase mutant phenotypes of *Aspergillus nidulans*, *A. fumigatus*, and *A. flavus*^a

Gene	Conidiation ^b	Resting structure ^c	Secondary metabolism ^d	Pathogenicity	Other	Source
<i>A. nidulans</i>						
<i>ΔppoA</i>	Slightly increased	Reduced	Slightly reduced	NA	...	Tsitsigiannis et al. 2004, 2005b, 2006.
<i>ΔppoB</i>	Increased	Reduced	Increased	Increased conidiation and maceration	Increased lipase activity	Tsitsigiannis et al. 2005b, 2006.
<i>ΔppoC</i>	Reduced	Increased	Increased	NA	...	Tsitsigiannis et al. 2004, 2005b, 2006.
<i>ΔppoA, ΔppoB, ΔppoC</i>	Reduced	Increased	ST reduced, Penicillin increased	Reduced sporulation and ST	Reduced lipase activity	Tsitsigiannis et al. 2005b, 2006.
<i>A. fumigatus</i>						
<i>ΔppoC</i>	Reduced	None	NT	Reduced	Aberrant conidial attachment	Dagenais et al. 2008.
<i>ppoA, ppoB ppoC IRT</i>	NA	None	NA	Increased (Murine model)	...	Tsitsigiannis et al. 2005a
<i>A. flavus</i>						
<i>ΔppoA</i>	NA for most part	NA for most part	NA	NA	...	This study
<i>ΔppoC</i>	Reduced	Increased	NA	NA	...	This study
<i>ΔppoD</i>	Increased	Reduced	NA	NA	...	This study
<i>lox</i>	Reduced	Increased	NA	NA	...	Horowitz et al. 2008
<i>IRT2</i>	Reduced	NA	NA	Reduced conidiation	Reduced lipase activity	This study
<i>IRT2-ΔppoC</i>	Reduced	Increased	NA	NT	...	This study
<i>IRT2-Δlox</i>	Reduced	Increased	NA	NT	...	This study
<i>IRT4</i>	Reduced	Increased	Increased	Reduced conidiation	...	This study

^a NA = no alteration and NT = not tested.

^b Conidiation assessed in fungal tissue culture.

^c Resting structure referred to the *A. nidulans* sexual stage cleistothecia or sclerotia in *A. flavus*. None = *A. fumigatus* has no resting structure.

^d Secondary metabolism refers to Sterigmatocystin (ST) and Penicillin among *A. nidulans* strains, gliotoxin for *A. fumigatus*, and aflatoxin (AF) for *A. flavus* strains.

Table 2. *Aspergillus flavus* strains used in this study

Strain ^a	Genotype	Source
NRRL 3357	Wild type	He et al. 2007
NRRL 3357.5	<i>pyrG</i>	He et al. 2007
TSHB2.39	<i>ΔAflx:pyrG; pyrG</i>	Horowitz et al. 2008
TSHB3.1.19	<i>ΔppoA:pyrG; pyrG</i>	This study
TRAW2.2.15	<i>ΔppoC:pyrG; pyrG</i>	This study
TJBS4.1.85	<i>ΔppoD:pyrG; pyrG</i>	This study
TJBS5.1.60	<i>ΔppoD:pyrG;niaD::ppoA, ppoB IRT:niaD</i>	This study
TSHB5.57	<i>ΔppoD:pyrG;niaD:ppoA, ppoB IRT:niaDppoC, lox IRT:phleomycin</i>	This study
TSHB6.3	<i>ΔppoD:pyrG;niaD:ppoA, ppoB IRT:niaDΔlox:pyrG</i>	This study
TSHB7.1	<i>ΔppoD:pyrG;niaD:ppoA, ppoB IRT:niaDΔppoC:pyrG</i>	This study
MJBS 4.0.50	<i>niaD-;ΔppoD:pyrG; pyrG</i>	This study
MTJBS5.1.60A	<i>PyrG-,PyrG- ΔppoD:pyrG;niaD:ppoA, ppoB IRT:niaD</i>	This study

^a Strains starting with T were original transformants and those starting with M resulted from chemical-induced selection.

contains the *A. parasiticus pyrG* marker gene surrounded by multiple cloning sites. The 5' and 3' flanking regions of *ppoA* were amplified using the primers *ppoA* 5flk Fwd (*SalI*), *ppoA* 5flk Rev (*EcoRI*) and *ppoA* 3flk Fwd (*BamHI*), *ppoA* 3flk Rev (*XbaI*), respectively. Each primer contains the engineered restriction site indicated. The 5' flank was inserted into pJW24 first with *SalI* and *EcoRI* to create pJBS3.0, followed by insertion of the 3' untranslated region using *BamHI* and *XbaI* to complete pJBS3.1. To create the *ppoD* disruption vector, 5' and 3' flanking regions of the gene were amplified using the primers *ppoD* 5flk Fwd (*SalI*), *ppoD* 5flk Rev (*EcoRI*) and *ppoD* 3flk Fwd (*BamHI*), *ppoB* 3flk Rev (*SpeI*), respectively. Each primer contains the engineered restriction site indicated. Initially, the 5' flank was inserted into pJW24 using *SalI* and *EcoRI* to create pJBS4.0. The 3' flank was inserted by *BamHI* and *SpeI* digest to create the final disruption vector, pJBS4.1. To create the *ppoC* disruption vector, flanking regions of the gene were amplified using the primers *ppoC* 5 flk Fwd (*SphI*) and *ppoC* 5flk Rev (*SalI*) for the 5' flanking region. The 3' flanking region of the gene was amplified by *ppoC* 3flk Fwd (*BamHI*) and *ppoC* 3flk Rev (*EcoRI*). Initially, the 5' flanking region was inserted by *SphI* and *SalI* into PUC18 vector (GenScript Corp., Piscataway, NJ, U.S.A.) containing a 2.8-kb *pyrG* cassette to create pRAW2.1. Then, the 3' flank was inserted by *BamHI* and *EcoRI* digest to create the final disruption vector, TRAW2.2.

niaD and *pyrG* auxotroph selection procedure.

To facilitate the creation of strains of *A. flavus* with multiple *ppo* deletions, it was necessary to create an auxotrophic marker in one of the *ppo* deletion strains. One isolate of *ΔppoD*

(TJBS4.1.85) was mutated to nitrate reductase auxotrophy following a modified form of the method outlined by Cove (1976). Briefly, 10⁵ conidia of TJBS4.1.85 were inoculated in 500 μl of sterile .01% Tween water evenly spread on 25 solid plates of fresh KCLO₃ medium. The cultures were incubated for 3 days at 29°C under constant light. Isolates with nonfunctional *niaD* are resistant to high concentration of KClO₃. Resistant isolates were transferred to GMM and ammonium GMM (GMM with 5 mM ammonium tartrate and no nitrate salts). Isolates that showed normal growth on 5 mM ammonium tartrate GMM and poor growth on GMM were screened on nitrogen test media. Ten of these isolates were screened for positive growth on four different nitrogen sources: 5 mM ammonium tartrate, 10 mM sodium nitrate, 10 mM sodium nitrite, and 100 mM hypoxanthine. Isolates with a nonfunctional *niaD* (*niaD*⁻) grew normally on each of the four nitrogen sources except sodium nitrate, where they were visibly reduced in diameter growth and conidiation. Of the 10 isolates screened on the nitrogen sources, 3 were *niaD*⁻ mutants. None of the three *niaD*⁻ mutants had apparent growth defects compared with NRRL 3357 when growing on non-nitrate media. One *niaD*⁻ mutant, MJBS 4.0.50, was selected for RNAi silencing of *ppoA* and *ppoB* by transformation with pJBS5.1. The resulting *AppoD*, IRT *ppoA*, *ppoB* triple mutant was designated TJBS5.1.60 and commonly referred to as IRT2 in this article. Later, IRT2 was used to generate a uracil auxotroph (a *pyrG*⁻ mutant) designated MTJBS5.1.60A, following the method outlined by He and associates (2007). MTJBS5.1.60A was used to create two more strains, IRT2-*ΔppoC* and IRT2-*Δlox*, using the TRAW2.2 construct for *ppoC* deletion or pSHB2 construct for *lox* deletion (Horowitz et al. 2008), both carrying the

Table 3. Oligonucleotides used in this study

Name	Sequence 5'–3'
ppoA 5flk Fwd	CCTCAGCCGTCGACTCGTATAC
PpoA 5flk Rev	CCTGAATTCATTGTTAGACGTGCTCG
PpoA 3flk Fwd	CCGCTCGGATTCTGACTGTAAGTTTA
PpoA 3flk Rev	TCCAACAGTAATCTAGAAAAGTCGAG
PpoA Int Fwd	CCCCGGTTTCGTATGTTGTTTGATA
PpoA Int Rev	TCCTCAACGCTGTCCTCAAAGATT
PpoA Fusion Fwd	GAAATGAAGCTTGGATGGCGCGCGAAGCAGCCGCTCAGGCCAC
PpoA Fusion Rev	GGTTGCCCCAAGCCGTGGGAAGTGGTGATCATATAGTCGCTTGAGC
PpoB 5flk Fwd	CTACCGAAGTCGACCCAGAGATCCTCGCGC
PpoB 5flk Rev	CAGGGCAAGGTTGGGTTATTGTAGAATTCGTCAG
PpoB 3flk Fwd	GTAGATGGATCCAAATCATTACAGATCGCGC
PpoB 3flk Rev	TACTTCCAGCATTTACGTAGAATCCACGGA
PpoB Int Fwd	TACGATAATGACCTTTTCCAGACG
PpoB Int Rev	TATATTCCTTCTGTTCCGGTGTC
PpoB Fusion Fwd	CGAGTTCGCTGATATACTAGTGGTCAAGGGTGCCGAAACCCGTTGG
PpoB Fusion Rev	ACACTGCCCGGGAGACGGTGGATCCTGGAGGCGCGCCAACA
PpoC 5flk Fwd	GAAACAAAGCAAAATGGCATGCAAAATGGCGCTC
ppoC 5flk Rev	GTTCCCTCGCAGCATCGAACAGG
ppoC 3flk Fwd	GGCTCCCTTAATGGATCCCGCAAG
ppoC 3flk Rev	GGTAAAGACTCCGCGCGCGAATTCTCCTTGG
ppoD 5flk Fwd	GACTTGTCGACCTTCTCCCATTTTC
PpoD 5flk Rev	CTTGAATTCGGGAACGTTTCTCGCAT
ppoD 3flk Fwd	GCGTGTAAGGATCCCGATGCTGTC
PpoD 3flk Rev	TATTTATTGTACTAGTCATCTGATCTTG
ppoD Int Fwd	TCCGTGTTATTGAGATTCGGGTA
PpoD Int Rev	AACGATTCCCTTTCCAGATTTCTC
NiaD Fwd	TGCACCTGTTATTATTAGTCTTACT
NiaD Rev	AGAGAAGCGCTACATGTGTATT
Lox Int Fwd	AGCCCCGATGAGCAGTTGAGT
Lox Int Rev	GTTACCTTTACGGCTCCCTCT
Actin Int Fwd	ACAGTCCAAGCGTGGTATCC
Actin Int Rev	GAAGCGGTCTGAATCTCCTG
ppoC Fusion Fwd	AACTTACCCTGGTATTGTTGCTGAGG
PpoC Fusion Rev	GTCTAAGGGCAGGTGGTCTGTTACTGGGATGGAGACGCTGGTAGAACCTGAGAATAGGTTA
Lox Fusion For	GTCTCCATCCCAGTAACGACCACCTGCCCTTAG
Lox Fusion For	TCTCAACGAACACGCTGGTGAAGTCTTCAATGG

pyrG as selectable marker. The resulting strains, TSHB6.3 and TSHB7.1, were designated IRT2- Δlox and IRT2- $\Delta ppoC$ through the article.

Plasmids constructions

to silence the *ppoA*, *ppoB*, *ppoC*, and *lox* expression.

pTMH44.2, containing the *A. nidulans gpdA* promoter and *trpC* terminator separated by approximately 280 bp of green fluorescent protein (*gfp*) with small multiple cloning sites upstream and downstream of *gfp* (McDonald et al. 2005), was utilized to construct both *ppoA::ppoB* RNAi and *ppoC::lox* RNAi vectors. Gene fragments from *ppoA* and *ppoB*, approximately 500 bp in length with no predicted introns, were amplified using the following primers (Table 3): *ppoA* fusion Fwd (5' *ppoA* *HindIII*/*NcoI*), *ppoA* fusion Rev (5' 23-bp *ppoB*, 23-bp *ppoA* 3'), *ppoB* Fusion Fwd (5' 23-bp *ppoA*, 23-bp *ppoB* 3'), and *ppoB* Fusion Rev (3' *ppoB* *BamHI*/*NcoI*). The two fragments of *ppoA* and *ppoB* were fused by PCR using methods described by Yu and associates (2004) and subcloned into pCR2.1 TOPO vector (Invitrogen). The resulting fusion fragment contained engineered restriction sites: 5'*ppoA* *HindIII*/*NcoI* and 3'*ppoB* *AscI*/*BamHI*. The nested sites *NcoI* and *AscI* were used to insert the fusion fragment in the forward orientation upstream of the *gfp* spacer fragment in pTMH44.2 to create pJBS5. Next, the outer restriction sites, *HindIII* and *BamHI*, were used to insert the fusion fragment in the reverse orientation to create pJBS5.1, which was sequenced through the region where each *ppo* fusion fragment meets pTMH44.2 (to confirm the orientation of each fusion fragment). For the *ppoC::lox* RNAi construct, gene fragments from *ppoC* and *lox*, approximately 800 and 700 bp, respectively, in length with no predicted introns, were amplified using the primers *ppoC* fusion Fwd (5' *ppoC*) and *ppoC::lox* fusion Rev (25-bp 3' *ppoC* and 44-bp 5' *lox*) for the *ppoC* fragment. The pair of primers *lox.fusion.for* Fwd (5' *lox*) and *lox.fusion.rev* (3' *lox*) were used to amplify the *lox* fragment. The two fragments of *lox* and *ppoC* were fused by PCR using methods described by Yu and associates (2004), then amplified by nested primers *ppoC.nest.notI.ncoI.for* and *lox.nest.BamHI.AscI.rev*. The resulting fusion fragment, *ppoC::lox*, contained engineered restriction sites at the 5' *ppoC* *notI*/*NcoI* and 3' *lox* *BamHI*/*AscI* ends. The nested sites *NcoI* and *AscI* were used to insert the fusion fragment in the forward orientation upstream of the *gfp* spacer fragment in pTMH44.2 to create pSHB6.8. Next, the outer restriction sites *BamHI* and *NotI* were used to insert the fusion fragment in the reverse orientation to create pSHB7.2, which was sequenced through the region where each *ppo* fusion fragment meets pTMH44.2. Plasmids pJBS5.1 and pSHB7.2 were used for transformation of MJBS4.0.50 and TJBS5.1.60 to silence the expression of *ppoA::ppoB* and *ppoC::lox*, respectively.

Fungal transformation procedure and mutants confirmation.

Fungal transformation essentially followed that of Miller and associates (1985). The *ppo* deletion strains were obtained by a double-crossover event, exchanging the *pyrG* selectable marker gene for the *ppo* coding region (Fig. 2A through C). *Aspergillus* protoplasts were produced from freshly germinated conidia from 3-day-old cultures and transformed using a polyethylene glycol method (Miller et al. 1985). The DNA (5 to 10 μ g) used for transformation was PCR amplified with nested primers from each disruption vector and gel purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Transformation with the *ppoA::ppoB* IRT vector pJBS5.1 was performed as a cotransformation with the *niaD* PCR product from *A. flavus* (primers listed in Table 3). Like-

wise, transformation with the *ppoC::lox* IRT vector pSHB7.2 was performed as a cotransformation with the pBC-Phleo plasmid carrying the phleomycin resistance cassette in which the *ble* gene is under the control of the *A. nidulans gpdA* promoter and the *Saccharomyces cerevisiae* CYC1 terminator (He et al. 2007). Transformants were screened for appropriate growth properties: uridine and uracil prototrophs for strains carrying a *pyrG* marker, nitrate prototrophs for strains with *niaD*, or resistance to phleomycin for strains carrying *ble*. Dot blots were made by spotting 3 μ l of genomic DNA from each transformant in a 1-cm grid on Hybond-XL membranes (Amersham Biosciences) and probed with an internal fragment of the appropriate *ppo* gene. Southern analysis performed in triplicate for each transformant was used to confirm a single gene replacement event in TJBS3.1.19, TJBS4.1.85, and TSHB2.2.15 (Fig. 2A through C), probing each transformant with the 5' flank and 3' flank or the ORF of the appropriate *ppo*. Strains carrying the *ppoA::ppoB* IRT vector pJBS5.1 were screened by dot blot in a fashion similar to *ppo* knockouts, and probed with the *gfp* spacer fragment excised from pTMH44.2 using *AscI* and *BamHI*. Spotted isolates with strong *gfp* hybridization were then selected for Southern analysis and probed with the same *gfp* fragment. Additionally, to confirm the efficiency of *ppoA* and *ppoB* transcript reduction, Northern analysis was performed using internal fragments of each gene from primers *ppoA* Int For, *ppoA* Int Rev, *ppoB* Int For, and *ppoB* Int Rev (Table 3). IRT4 strains carrying the *ppoC::lox* IRT vector in addition to the *ppoA::ppoB* IRT vector were confirmed by digestion with *NcoI* and were screened for the presence of a 3.2-kb band and contain the inverted repeat transgene. The *ppoC::lox* IRT strain carrying a single copy was called TSHB5.57. Northern analysis was performed using a DNA probe of internal fragments of each gene from primers *ppoC* Int For, *ppoC* Int Rev, *lox* Int For and *lox* Int Rev, *ppoA* Int For, *ppoA* Int Rev, and ActinF and ActinR for Actin-specific DNA probe (Table 3). Detection of signals was carried out with a Phosphorimager-SI (Molecular Dynamics).

Statistical analysis.

Data were analyzed using the JMP software package (version 3.2.6; SAS Institute, Inc, Cary, NC, U.S.A.). Mean comparisons of sclerotia and conidial formation affected by different conidia concentration or strains were calculated using least significant difference according to the Tukey-Kramer multiple comparison test at $P \leq 0.05$. Mean values with different letters are significant.

ACKNOWLEDGMENTS

We thank G. A. Payne for access to *A. flavus* genome data and J. Starr for peanut seed. This research was funded by BARD No. FI-384-2006 to S. H. Brown and National Science Foundation IOB-0544428, subagreement S060039 to N. P. Keller.

LITERATURE CITED

- Bennett, J. W. 1979. Aflatoxin and anthraquinones from diploids of *Aspergillus parasiticus*. J. Gen. Microbiol. 113:127-136.
- Bhatnagar, D., Cary, J. W., Ehrlich, K., Yu, J., and Cleveland, T. E. 2006. Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. Mycopathologia 162:155-166.
- Brodhagen, M., Tsitsigiannis, D. I., Hornung, E., Goebel, C., Feussner, I., and Keller, N. P. 2008. Reciprocal oxylipin-mediated cross-talk in the *Aspergillus*-seed pathosystem. Mol. Microbiol. 67:378-391.
- Burow, G. B., Nesbitt, T. C., Dunlap, J., and Keller, N. P. 1997. Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis. Mol. Plant-Microbe Interact. 10:380-387.
- Burow, G. B., Gardner, H. W., and Keller, N. P. 2000. A peanut seed lipoxygenase responsive to *Aspergillus* colonization. Plant Mol. Biol. 42:689-701.

- Calvo, A. M., Hinze, L. L., Gardner, H. W., and Keller, N. P. 1999. Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. *Appl. Environ. Microbiol.* 65:3668-3673.
- Calvo, A. M., Wilson, R. A., Bok, J. W., and Keller, N. P. 2002. Relationship between secondary metabolism and fungal development. *Microbiol. Mol. Biol. Rev.* 66:447-459.
- Calvo, A. M., Bok, J. W., Brooks, W., and Keller, N. P. 2004. VeA is required for toxin and sclerotial production in *Aspergillus parasiticus*. *Appl. Environ. Microbiol.* 70:4733-4739.
- Champe, S. P., and El-Zayat, A. A. 1989. Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. *J. Bacteriol.* 171:3982-3988.
- Champe, S. P., Rao, P., and Chang, A. 1987. An endogenous inducer of sexual development in *Aspergillus nidulans*. *J. Gen. Microbiol.* 133:1383-1387.
- Chen, H., and Fink, G. R. 2006. Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev.* 20:1150-1161.
- Chen, H., Fujita, M., Feng, Q., Clardy, J., and Fink, G. R. 2004. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc. Natl. Acad. Sci. U.S.A.* 101:5048-5052.
- Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* 31:3497-3500.
- Cotty, P. J. 1988. Aflatoxin and sclerotial production by *Aspergillus flavus*: Influence of PH. *Phytopathology* 78:1250-1253.
- Cotty, P. J., and Mellon, J. E. 2006. Ecology of aflatoxin producing fungi and biocontrol of aflatoxin contamination. 22:110-117.
- Cove, D. J. 1976. Chlorate toxicity in *Aspergillus nidulans*: The selection and characterization of chlorate resistant mutants. *Heredity* 36:191-203.
- Dagenais, T., Chung, D., Giles, S., Hull, C., Andes, D., and Keller, N. P. 2008. Defects in conidiophore development and conidial/macrophage interactions in a dioxygenase mutant of *Aspergillus fumigatus*. *Infect. Immun.* 76:3214-20.
- Diener, U. L., Cole, R. J., Sanders, T. H., Payne, G. A., Lee, L. S., and Klich, M. A. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annu. Rev. Phytopathol.* 25:249-270.
- Funk, C. D. 2001. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* 294:1871-1875.
- Gao, X., Shim, W. B., Göbel, C., Kunze, S., Feussner, I., Meeley, R., Balint-Kurti, P., and Kolomiets, M. 2007. Disruption of a maize 9-lipoxygenase results in increased resistance to fungal pathogens and reduced levels of contamination with mycotoxin fumonisin. *Mol. Plant-Microbe Interact.* 20:922-933.
- Gao, X., Brodhagen, M., Isakeit, T., Horowitz Brown, S., Gobel, C., Betran, J., Feussner, I., Keller, N. P., and Kolomiets, M. V. 2009. Inactivation of the lipoxygenase ZmLOX3 increases susceptibility of maize to *Aspergillus* spp. *Mol. Plant-Microbe Interact.* 22:222-231.
- Garscha, U., Jerne, F., Chung, D. W., Keller, N. P., Hamberg, M., and Oliu, E. H. 2007. Identification of dioxygenases required for *Aspergillus* development: Studies of products, stereochemistry and the reaction mechanism. *J. Biol. Chem.* 282:34707-34718.
- Gqaleni, N., Smith, J. E., Lacey, J., and Gettinby, G. 1997. Effect of temperature, water activity, and incubation time on production of aflatoxins and cyclopiazonic acid by an isolate of *Aspergillus flavus* in surface agar culture. *Appl. Environ. Microbiol.* 63:1048-1053.
- Greene, A. V., Keller, N. P., Haas, H., and Bell-Pedersen, D. 2003. A circadian oscillator in *Aspergillus* spp. regulates daily development and gene expression. *Eukaryot. Cell* 2:231-237.
- Guzmán-de-Peña, D., and Ruiz-Herrera, J. 1997. Relationship between aflatoxin biosynthesis and sporulation in *Aspergillus parasiticus*. *Fungal Genet. Biol.* 21:198-205.
- He, Z. M., Price, M. S., Obrian, G. R., Georgianna, D. R., and Payne, G. A. 2007. Improved protocols for functional analysis in the pathogenic fungus *Aspergillus flavus*. *BMC Microbiol.* 7:104.
- Hedayati, M. T., Pasqualotto, A. C., Warn, P. A., Bowyer, P., and Denning, D. W. 2007. *Aspergillus flavus*: Human pathogen, allergen and mycotoxin producer. *Microbiology* 153:1677-1692.
- Horn, B. W. 2007. Biodiversity of *Aspergillus* section Flavi in the United States: A review. *Food Addit. Contam.* 24:1088-1101.
- Horowitz Brown, S., Zarnowski, R., Sharpee, W. C., and Keller, N. P. 2008. Morphological transition governed by density dependent and lipoxygenase activity in *Aspergillus flavus*. *Appl. Environ. Microbiol.* 74:5674-5685.
- Hyeon, B. 1976. Chemical studies on the factors controlling sporulation of fungi. *Chem. Regul. Plants* 11:69-76.
- Keller, N. P., and Hohn, T. M. 1997. Metabolic pathway gene clusters in filamentous fungi. *Fungal Genet. Biol.* 21:17-29.
- Klich, M. 2007. *Aspergillus flavus*: The major producer of aflatoxin. *Mol. Plant Pathol.* 8:713-722.
- Liavonchanka, A., and Feussner, I. 2006. Lipoxygenases: Occurrence, functions and catalysis. *J. Plant Physiol.* 163:348-357.
- McDonald, T., Devi, T., Shimizu, K., Sim, S.-C. and Keller, N. P. 2004. Signaling events connecting mycotoxin biosynthesis and sporulation in *Aspergillus* and *Fusarium* spp. Pages 139-147 in: *New Horizon of Mycotoxicology for Assuring Food Safety*. T. Yoshizawa, ed. Bikhosha. Co., Takamatsu, Japan.
- McDonald, T., Brown, D., Keller, N. P., and Hammond, T. M. 2005. RNA Silencing of mycotoxin production in *Aspergillus* and *Fusarium* species. *Mol. Plant-Microbe Interact.* 18:539-545.
- Miller, B. L., Miller, K. Y., and Timberlake, W. E. 1985. Direct and indirect gene replacements in *Aspergillus nidulans*. *Mol. Cell. Biol.* 5:1714-1721.
- Nemchenko, A., Kunze, S., Feussner, I., and Kolomiets, M. 2006. Duplicate maize 13-lipoxygenase genes are differentially regulated by circadian rhythm, cold stress, wounding, pathogen infection, and hormonal treatment. *J. Exp. Bot.* 57:3767-3779.
- Nukima, M., Sassa, T., Ikeda, M., and Takahashi, K. 1981. Linoleic acid enhances perithecial production in *Neurospora crassa*. *Agric. Biol. Chem.* 45:2371-2373.
- Robens, J., and Cardwell, K. 2003. The cost of mycotoxin management to the USA: Management of aflatoxins in the United States. *J. Toxicol. Toxin Rev.* 22:143-156.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A..
- Scheidegger, K. A., and Payne, G. A. 2003. Unlocking the secrets behind secondary metabolism: A review of *Aspergillus flavus* from pathogenicity to functional genomics. *J. Toxicol. Toxin Rev.* 22:423-459.
- Shea, J. M., Henry, J. L., and Del Poeta, M. 2006. Lipid metabolism in *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* 6:469-479.
- Shimizu, K., and Keller, N. P. 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* 157:591-600.
- Sprague, G. F., and Winans, S. C. 2006. Eukaryotes learn how to count: Quorum sensing by yeast. *Genes Dev.* 20:1045-1049.
- Tsitsigiannis, D. I., and Keller, N. P. 2006. Oxylinins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans*. *Mol. Microbiol.* 59:882-892.
- Tsitsigiannis, D. I., and Keller, N. P. 2007. Oxylinins as developmental and host-fungal communication signals. *Trends Microbiol.* 15:109-118.
- Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R., and Keller, N. P. 2004. Endogenous lipogenic regulators of spore balance in *Aspergillus nidulans*. *Eukaryot. Cell* 3:1398-1411.
- Tsitsigiannis, D. I., Bok, J. W., Andes, D., Nielsen, K. F., Frisvad, J. C., and Keller, N. P. 2005a. *Aspergillus* cyclooxygenase-like enzymes are associated with prostaglandin production and virulence. *Infect. Immunol.* 8:4548-4559.
- Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R., and Keller, N. P. 2005b. Three putative oxylinin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans*. *Microbiology* 151:1809-1821.
- Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Dominguez, Y. R., and Scaccocchio, C. 2004. Double-joint PCR: A PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genet. Biol.* 41:973-981.
- Wicklow, D. T., Horn, B. W., and Cole, R. J. 1982. Sclerotium production by *Aspergillus flavus* on corn kernels. *Mycologia* 74:398-403.

AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information Conserved Domain search page: www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
 San Diego Supercomputer Center Biology Workbench tool: workbench.sdsc.edu
 Image J software: rsb.info.nih.gov/ij/index.html
 EMBL-EBI ClustalW multiple sequence alignment program: www.ebi.ac.uk/clustalw
 The Center for Integrated Fungal Research *Aspergillus flavus* website: www.aspergillusflavus.org