A Caleosin-Like Protein with Peroxygenase Activity Mediates Aspergillus flavus Development, Aflatoxin Accumulation, and Seed Infection

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Caleosins are a small family of calcium–binding proteins endowed with peroxygenase activity in plants. Caleosin-like genes are present in fungi; however, their functions have not been reported yet. In this work, we identify a plant caleosin-like protein in Aspergillus flavus that is highly expressed during the early stages of spore germination. A recombinant purified 32-kDa caleosin-like protein supported peroxygenase activities, including co-oxidation reactions and reduction of polyunsaturated fatty acid hydroperoxides. Deletion of the caleosin gene prevented fungal development. Alternatively, silencing of the gene led to the increased accumulation of endogenous polyunsaturated fatty acid hydroperoxides and antioxidant activities but to a reduction of fungal growth and conidium formation. Two key genes of the aflatoxin biosynthesis pathway, aflR and aflD, were downregulated in the strains in which A. flavus PXG (AfPXG) was silenced, leading to reduced aflatoxin B1 production in vitro. Application of caleosin/peroxygenase-derived oxylipins restored the wild-type phenotype in the strains in which AfPXG was silenced. PXG-deficient A. flavus strains were severely compromised in their capacity to infect maize seeds and to produce aflatoxin. Our results uncover a new branch of the fungal oxylipin pathway and may lead to the development of novel targets for controlling fungal disease.

Oxylipins constitute a large family of diverse oxygenated fatty acids and derivatives present in mammals, plants, algae, and fungi (1–3). While the biosynthesis and the roles of animal and plant oxylipins have been extensively studied (4–8), knowledge about fungal oxylipins remains limited. Fungal oxylipins are widespread among filamentous fungi, yeasts, and oomycetes (9–11) and were first described to be precocious sexual inducers, or psi factors (12). They are composed of a mixture of hydroxylated oxylipins derived from oleic (18:1), linoleic (18:2), and linolenic (18:3) acids under the action of psi factor-producing oxygenase (Ppo) enzymes (13–15). Linoleate diol synthase (LDS) converts linoleic acid directly to hydroxylated derivatives (8). However, most of the fungal oxylipins derive from an initial hydroperoxidation step, whereby polyunsaturated fatty acids (PUFAs) are catalyzed by lipoxygenases (LOXs) and dioxygenases (DOXs) (8, 16, 17). Whereas in plants such enzymes form essentially three types of hydroperoxides (OOHs), i.e., 9-OOH, 13-OOH, and 2-OOH, in fungi, they can introduce molecular oxygen on the carbon 8, 10, 11, or 15 of PUFA, yielding 8-OOH, 10-OOH, 11-OOH, and 15-OOH derivatives, respectively (8, 10, 11, 17, 18). Whatever their mode of formation, fatty acid hydroperoxides (FAOOHs) and their metabolites have been reported to play crucial roles in the life cycle of fungi, notably, in conidiogenesis and sclerotium formation (19). In addition, Ppo-derived psi factors produced by Aspergillus nidulans were shown to regulate both asexual and sexual spore development (12, 14, 20).

Fungal oxylipins are also involved in the regulation of secondary metabolism, involving the synthesis of mycotoxins and antibiotics. For example, deletion of Ppo enzymes yielded mutants depleted of the mycotoxin sterigmatocystin but enriched with penicillin (21). A lipoxygenase-like enzyme-deficient Aspergillus ochraceus strain producing low levels of linoleic acid-derived 13-hydroperoxyoctadecadienoic acid (13-HPOD) displayed decreased ochratoxin A production and delayed formation of conidia but increased production of sclerotia. Complementation of the culture medium with 9-HPOD and 13-HPOD enhanced the production of ochratoxin A in wild-type (WT) A. ochraceus but not in a mutant in which the LOX-like gene was deleted (19). Fungal production of aflatoxins seems to be favored by an oxidative environment. For example, the oxidative stress caused by the addition of cumene hydroperoxide and H2O2 was reported to induce aflatoxin accumulation (22). In contrast, plant-derived antioxidants diminished aflatoxin formation without affecting fungal growth (23). Besides endogenous oxylipins, several lines of evidence show that during the Aspergillus-seed interaction, the host oxylipins also play determinant roles in the biology of the fungus (24). For example, 13-HPOD affected sexual spore development in A. nidulans and Aspergillus flavus, whereas spore germination was inhibited by C9 to C11, derivatives of the phytooxylipin pathway (25). In addition, while 13-HPOD decreased the levels of production of mycotoxins, 9-HPOD did not reduce their levels of biosynthesis (26). Moreover, genetic evidence for reciprocal oxylipin cross talk during the plant–seed interaction has been...
reported: plant oxylipin directly or indirectly affected *Aspergillus* development processes, whereas plant oxylipin production, in turn, was modified during infection by the fungus (27). Interestingly, plant fatty acid hydroperoxides and their corresponding alcohols are metabolized by fungi into trihydroxy derivatives (28). In plants, such oxylipins are derived from the hydrolysis of 15,16-epoxy-13-hydroxy-9,11-octadecenoic acid. The formation of these quite unusual epoxy alcohols is catalyzed by peroxygenase (29). In addition to \( \alpha \)-dioxygenases and cytochrome P450 enzymes, this enzyme is known to initiate one of the branches of the phytooxylipin pathway. Such peroxygenases have been identified as caleosins, which constitute a small family of Ca\(^{2+}\) peroxides. Such peroxygenases have been identified to be caleosins, which constitute a small family of Ca\(^{2+}\) peroxides. These peroxygenases are reported: plant oxylipin directly or indirectly affected as described previously (37, 38) with a slight modification. Fungal mycelia from 7-day-old cultures were pelleted by centrifugation at 5,000 × g, and the cells were washed with 200 ml of washing buffer (50 mM Tris-HCl, pH 7.5). The pellet (10 g) was resuspended in 30 ml of the same buffer containing 0.6 M sorbitol and 1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich) and kept on ice during the preparation procedure. The fungal cells were disrupted by a high-performance disperser (Ultra-Turrax; IKA, Germany). The resulting homogenate was centrifuged once at 10,000 × g for 15 min. The supernatant was recovered and centrifuged at 100,000 × g for 90 min. After centrifugation, a fine, floating, creamy layer of lipid droplets, corresponding to oleosomes, was obtained on the surface of the preparation, while the pellet corresponded to microsomes. The crude lipid droplet fraction was carefully collected with a pipette and washed with 100 mM potassium pyrophosphate buffer that contained 0.1 M sucrose (pH 7.4). After centrifugation at 100,000 × g for 45 min, the oleosomes were taken and finally suspended in 10 mM sodium acetate (0.1 M, pH 5.5). The reaction was stopped with a drop of 50 μl of purified recombinant AfPXG in a total volume of 500 μl. The protein concentrations in each fraction were estimated by a Bradford assay (Bio-Rad) using bovine serum albumin as a standard (39).

**Peroxynasme activities.** Peroxynasme activity was routinely measured during the purification procedure by use of aniline and thioenamide as the substrate (40). Epoxidation of \([1-14]C\)oleic acid and \([1-14]C\)linoleic acid was performed as described by Blee and Schuber (41). The hydroperoxide reductase activity of *Aspergillus flavus* PXG (APXG) was measured by incubation of 9-HPOD or 13-HPOD overnight at 26°C with 50 μg of purified recombinant APXG in a total volume of 500 μl of sodium acetate (0.1 M, pH 5.5). The reaction was stopped with a drop of HCl (4 N), and the residual substrate and products were extracted three times in 2 ml of dichloromethane-ether (1:1, vol/vol). After the extracts were dried under a nitrogen flow, the extracts were taken with 25 μl of acetonitrile-water-acetic acid (50:50:0.1, vol/vol/vol). The extracts were analyzed using a Jasco LC-2000 Plus series high-pressure liquid chromatography (HPLC) system (Jasco, USA), a UV detector (234 nm; RF-10Axl; Shimadzu), and a C\(_18\) column (150 by 4.6 mm; particle size, 5 μm; Eclipse

**MATERIALS AND METHODS**

**Materials, chemicals, host strains, and culture conditions.** Oligonucleotides were provided either by Eurofins or by Sigma–France. Aniline, thioenamide, cumene hydroperoxide, and aflatoxin B1 (AFB1) and AFB2 were purchased from Sigma-Aldrich. \([1-14]C\)oleic acid and \([1-14]C\)linoleic acid (52 and 50 μCi/mmol, respectively) were purchased from PerkinElmer Life Sciences. *Aspergillus flavus* strain FSS63, isolated from a local farmed soil, was morphologically and biochemically identified by the Laboratory of Microbiological Enzymes of the Atomic Energy Commission of Syria (AECS). This strain was subjected to a fine molecular characterization. Using primers designed to amplify the intergenic transcribed spacer (ITS) region as described by White et al. (34), the ITS region was amplified and sequenced. Moreover, the identity of this strain was confirmed using a protocol proposed by Godet and Munaut (35) as well as by the restriction fragment length polymorphism (RFLP) method as described before (36). The primers used in this study are presented in Table 1. Stock cultures of *A. flavus* were maintained in slant tubes at 4°C on potato dextrose agar (PDA; Difco Laboratories, USA). For solid or liquid cultures of *A. flavus*, the stock culture was transferred onto petri dishes containing PDA or into a 500-ml Erlenmeyer flask containing 100 ml of potato dextrose (PD) broth (Difco Laboratories, USA). For solid or liquid cultures of *A. flavus*, the stock culture was transferred onto petri dishes containing PDA or into a 500-ml Erlenmeyer flask containing 100 ml of potato dextrose (PD) broth, respectively, and allowed to develop for 7 days at 28°C. *Escherichia coli* strain TOP10 was used as the host for plasmid cloning experiments. Bacteria were grown in Luria-Bertani medium supplemented with ampicillin (100 μg ml\(^{-1}\)) at 37°C. *Saccharomyces cerevisiae* strain W6 (ade his7-2 leu2-3 leu2-112 ura3-52) was used as the host for protein expression. Recombinant yeast was grown in S medium (7 g liter\(^{-1}\) yeast nitrogen base, 1 g liter\(^{-1}\) Casamino Acids, and 20 g liter\(^{-1}\) glucose supplemented with 50 μg ml\(^{-1}\) histidine, 200 μg ml\(^{-1}\) adenine, and 50 μg ml\(^{-1}\) leucine) for 2 days with shaking at 30°C.

| Table 1 Names and nucleotide sequences of primers used in this study* |
|-----------------------------|-----------------------------|
| Name | Oligonucleotide sequence (5′–3′) |
| IT51 | TCGTGAATTCTAGCCTGCG |
| IT54 | TCCTCGCCGTATGATATGC |
| AflbR | GCACAAATGGCTTCTTCGT |
| AflbRF | CCGCGGAAGACATCCTTTAC |
| AflbR | GAGCCCACTTCCGAAATAC |
| AflPXGqF | ATGCATTACCGCGAGGCTTC |
| AflPXGqR | GCAGGCTTATACGAATATC |
| AflPXGqF | UGGGCAAAAGGUGCCGGGU |
| AflPXGqR | CCCGGACCUUAUGGCCCAAU |
| AflPXGq2F | GCUAGCAGCGCCGGACGU |
| AflPXGq2R | GUGGAGCGCCGUAGCCGU |
| P1 | ATGGTGGTGAATGATATCA |
| P2 | GAGTTCAGGCTTTTTTCTATATATGATATC |
| P3 | CCGAGGCGAAGGAGTAGGAGCCGGTGTGATAC |
| P4 | TCTTTGTACACGGTATAC |
| P5 | TTGATTTATAATTTAGTTATGAAAAGCTGGAATC |
| P6 | AAATGATGTAACCGCGCCCTATTTTGGGCCCTGG |

* Underlined nucleotides indicate BamHI and HindIII restriction sites. Poly-His codons were inserted at the N terminus of primer AflPXG.NHis (6× CAC) and the C terminus of AflPXG.CH (6× GTG).
Expression of recombinant APXG protein. APXG was subcloned into the yeast constitutive expression vector pVT102U (42) using the BamHI-HindIII site. The correct recombinant plasmid, pVT102U/APXG, was then introduced into the yeast Saccharomyces cerevisiae W66 (ade his2-2 leu2-3 leu2-112 ura3-52) (43). Expression of recombinant APXG in transformed yeast cells was carried out as described by Hanano et al. (30). The microsomes of the recombinant yeasts were resuspended in 10 mM potassium phosphate (pH 8) containing 10% (vol/vol) glycerol and were treated with Emulphpoll (final concentration, 0.2%) for 45 min at 4°C. The mixture was centrifuged at 100,000 × g for 2 h.

Solubilization and purification of recombinant APXG. All the subsequent steps were performed on ice (4°C). The oleosomes of recombinant yeasts resuspended in 10 mM potassium phosphate (pH 8) containing 10% (vol/vol) glycerol were treated separately with different detergents (CHAPS [3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate], Triton X-100, Emulphpoll, Sigma) at a final concentration of 0.2% (vol/vol) for 45 min. The mixture was then centrifuged at 100,000 × g for 45 min. After centrifugation, the supernatant was taken, the concentration of the solubilized proteins was estimated by the Bradford assay (39), and enzymatic activity was determined. The solubilized fraction was used in the protein purification procedure. The His-tagged APXG present in the supernatant was purified on an Ni-nitrilotriacetic acid Superflow column (Qiagen) under native (non-denaturing) conditions, according to the manufacturer’s instructions. The purity of the recombinant protein was confirmed by 12% SDS-PAGE, followed by Coomassie blue staining. For Western blot experiments, proteins were fractionated by 12% SDS-PAGE and electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a mini-Transblot cell transfer apparatus (Bio-Rad). For detection of PXG-His, a mouse monoclonal anti-His antibody and an anti-mouse immunoglobulin antibody conjugated to peroxidase were used at 1:2,000. Blots were developed using an enhanced chemiluminescence kit from Pierce.

Replacement of the PXG-like gene by an Hyg 

Design of siRNAs. Two siRNAs that targeted the mRNA sequence of the PXG-like gene by the hygromycin resistance (Hyg) gene was carried out as described by Ninomiya et al. (44). The 5′ and 3′ flanking DNA (1 kb) of the PXG-like gene was amplified by PCR using the genomic DNA of A. flavus FSS63 as a template. The primers used for the amplification of the 5′ or 3′ flanking DNA were primers P1 and P2 or primers P3 and P4, respectively (Table 1). The Hyg' gene was amplified by PCR using the binary vector pcCAMBA1381 (http://www.cambia.org/data/cambia/585.html) (GenBank accession number AF234302) as a template and primers P5 and P6. The three PCR products were mixed and used as a template in a fusion PCR (45). The fusion PCR product was analyzed on an agarose gel (1%) and used for transformation of the protoplasts of the A. flavus FSS63 wild type.

Preparation of fungal protoplast and transformation. Protoplasts were prepared from fungal conidia as described by Cheng and Belanger (46) with some modifications. Briefly, 7-day-old cultures of A. flavus were obtained on PDA plates. The expected conidia (about 1 × 10⁶ conidia per plate) were harvested and then washed with sterile water. The spores were suspended in 20 ml of a solution of 25 mM l-mercaptoethanol and 5 mM Na₂EDTA, pH 8.0, under gentle shaking for 20 min at room temperature. The treated spores were centrifuged for 5 min at 5,000 rpm, and the pellets were resuspended and incubated for 2 h at 25°C in 5 ml of...
gene, a solution (5 μl) was spread on agar medium containing hygromycin B (500 μg/ml) was added, and the components were gently mixed. The tubes were incubated at 20°C for 24 h to allow transfection to proceed. Then, the mixture was inoculated in 10 ml of PD medium with 1.2 M sorbitol for 7 days at 28°C in the dark. Different dilutions of siRNA (25, 50, 100 nM) were tested on A. flavus. A similar treatment of protoplasts without siRNA or with nonspecific siRNA was performed as a negative control. All experiments were carried out using three biological replicates. For replacement of the PXG-like gene, a solution (5 μl) containing 10 μg DNA of the fusion PCR product was mixed with 50 μl of protoplasts, and the mixture was incubated on ice for 5 min. Forty microliters of the mixture was transferred to an electroporation cell (BTEX Electroporation, 600 V; Genetronics) and shocked by using a charging voltage of 1.5 kV and a resistance of 186 Ω. After the electroshock, 1 ml of PD medium containing 1.2% sucrose was added and the conidia were incubated at 30°C for 2 h. The solution (200 μl) was spread on agar medium containing hygromycin B (500 μg/ml). Colonies resistant to hygromycin were isolated and tested by PCR using primers P1 and P6. Southern blotting was used to confirm whether the colonies contained extra copies of the Hpy’ gene.

Biomass and conidium number measurements. The fungal biomass of 7-day-old cultures on PDA plates was determined. The transformants or the control strain was inoculated at a single point on a cellophane membrane placed on the surface of a PDA plate. Seven days later, the membranes were removed and the corresponding mycelial mats were collected, thoroughly washed twice with distilled water, filtered through Whatman no. 4 filter papers, and dried overnight in an oven at 95°C. Mycelial dry weights were then determined as described by Rasooli and Razzaghi-Abyaneh (48). Fungal growth inhibition (in percent) was calculated according to the following formula: [(total control weight – total sample weight)/total control weight] × 100. In parallel, the total conidium were harvested from each plate and placed in 5 ml of water containing 0.01% Tween 80. The conidia were diluted to 1:10 and counted with a hemocytometer.

Extraction, cleanup, and HPLC analysis of aflatoxin. One milliliter of the conidial suspension (1 × 10⁷ spores/ml) generated from A. flavus was cultivated on petri plates containing PDA. The plates were incubated at 28°C for 7 days. The total growth of the fungi was collected to extract the aflatoxins (AFs). The extraction of AFs produced by A. flavus was carried out as described by Bertuzzi et al. (49) by placing the A. flavus fungi in 100 ml of chloroform and rotating the mixture on a rotary shaker for 1 h. The cleanup of the extract was done as described previously (50) using a thin-layer chromatography (TLC) plate. Extracted AF samples were spotted onto a silica reversed-phase TLC plate (aluminum sheets, 20 by 20 cm, 200-μm layer; Merck, Germany), and the chromatogram was developed using a solvent system of chloroform-acetone (90:10, vol/vol). After development, the spot with an Rf value similar to that of the AFB1 standard was scraped and then reextracted with chloroform and evaporated to dryness under nitrogen. The extract was resuspended with 100 μl acetonitrile and stored in an amber-colored vial under refrigeration. Extracts were analyzed using a Jasco LC-2000 Plus series HPLC system (Jasco, USA), a fluorescence detector (excitation λ, 247 nm; emission λ, 480 nm; RF-10AXll; Shimadzu), and a C18 column (150 by 4.6 mm; particle size, 5 μm; column temperature, 53°C; Eclipse XDB-C18; Agilent, USA). The analysis was performed using a mobile phase of water–methanol–acetonitrile (50:40:10, vol/vol/vol) at a flow rate of 0.8 ml min⁻¹ and a run time of 10 min. To study the time course of aflatoxin production in the strains in which A/PXG was silenced and with different treatments, aflatoxin production was evaluated at 2, 5, 7, 9, 11, and 13 days after inoculation.

SOD and CAT enzymatic activities. Preparation of the fungal tissues for determination of enzyme activities was carried out as described previously (51), with some modification. Briefly, 5 g of fungal tissues was homogenized with 5 ml potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM Na₂-dithiothreitol, and 5% (wt/vol) insoluble polyvinylpyrrolidone on ice. Subsequently, the homogenate was centrifuged at 12,000 rpm for 5 min, and the supernatant was used for analysis of the enzymatic activities. Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (52). Catalase (CAT) activity was measured by the method of Azevedo et al. (53). Activity was determined by monitoring the decrease in absorbance due to H₂O₂ reduction at 240 nm for 2 min.

Complementation of strains in which A/PXG was silenced with PXG pathway oxylipins. C18:2 Oxylipsins derived from the LOX-PXG pathway were biosynthesized in three separate steps. In the first step, hydroperoxides 9-HP0D and 13-HP0D were prepared by incubation of C18:2 with 9-LOX and 13-LOX from tomato and soybean, respectively, as described previously (54, 55). In the second step, two possible forms of epoxide, 9,10-epoxy-12(9)-octadecenoic acid (9,10-EOE) and 12,13-epoxy-9(13)-octadecenoic acid (12,13-EOE), were produced from linoleic acid by incubation of radiolabeled C18:2 (50 μCi/mmol in ethanol) overnight at 26°C in the presence of 50 μg of purified recombinant AFXG and 10 μl of cumene hydroperoxide (20 mM). Reaction, extraction, and purification of epoxides were carried out as described by Hanano et al. (30). Finally, the purified epoxides resulting from the second step were hydrolyzed to their corresponding dihydroxy compounds, 9,10-dihydroxy-12(9)-octadecenoic acid (9,10-DHOE) and 12,13-dihydroxy-9(13)-octadecenoic acid (12,13-DHOE). The hydrolysis of epoxides can take place chemically in acidic solution (pH 5.5). Then, the dihydroxy compounds were extracted, purified, and analyzed as described by Summarer et al. (56). An ethanol solution of 100 μM containing a mix of epoxides (mix-EOE) or dihydroxy compounds (mix-DHOE) was added to the liquid cultures of the (+) siRNA PXG1 strain in a 500-ml Erlenmeyer flask containing 100 ml of PD broth for 2 days at 28°C. To maximize any possible effects of these oxylipins on mutant growth, the mutant was first fed on PD broth containing a mix of 9-hydroxyoctadecadienoic acid (HOD) and 13-HOD (100 μM each) for 5 days at 28°C. Subsequently, a known quantity of fungal growth was then transferred onto petri dishes containing PDA supplemented with the oxylipins indicated below and allowed to develop for 5 days at 28°C. Six transformants of A. flavus were examined for their responses to each oxylipin. In parallel, the control experiment was carried out using ethanol only.

Inoculation of maize seeds with A. flavus and biomass estimation. A quantity of 100 g of maize (Zea mays) was sterilized by emerging maize grains into 70% ethanol for 1 min. After they were dried, the grains were placed in a sterile petri plate and directly inoculated with 200 μl of a liquid culture of A. flavus in PD broth. The inoculated grains were incubated at 28°C for 7 days. Fungal biomass was estimated on day 7 by careful washing of the infected grains, filtration, and then weighing of the fungal growth, expressed as the number of grains (fresh weight) per 100 g of grains.

Statistical analysis. All data presented are expressed as means ± standard deviations (SDs). Comparisons between control and treated strains were evaluated by the t test. The difference from the control was considered significant when P = <0.05, very significant when P = <0.01, and highly significant when P = <0.001.

Nucleotide sequence accession numbers. The sequences of the ITS region and the A/PXG gene of A. flavus were submitted to GenBank and may be found under accession numbers KC621105 and KJ668859, respectively.
**RESULTS**

Aspergillus flavus contains a single caleosin gene-like gene. When the sequence of the Arabidopsis thaliana At4g26740 gene, encoding the first characterized caleosin/peroxygenase (30), was compared with the sequence of the A. flavus NNRL 3357 genome by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi), it presented about 50% identity with the A. flavus AFLA_00285 gene. The DNA sequence of this gene, which has no biological functions known so far (57), contains an ORF of 873 nucleotides encoding a putative calcium-binding protein. Primers designed on the basis of the AFLA_00285 gene sequence (primers AfPXGF and AfPXGR; Table 1) were used to clone a homologous gene from toxigenic isolate A. flavus FSS63. An open reading frame of 873 nucleotides encoding a polypeptide of 291 amino acids (Gen-Bank accession number KJ668859) was identified in the cloned gene.

Multiple-sequence alignment of the protein sequences revealed that this fungal protein has a high degree of similarity (67%) with Arabidopsis caleosins (Fig. 1A). As expected for a caleosin, AFLA_00285 contains an EF-hand calcium-binding site motif near its N terminus and several putative phosphorylation sites in the C terminus (58). Like some plant caleosins, it lacks most of the conserved proline residues in the central hydrophobic region initially postulated to be essential for caleosin anchoring in membranes (59) (Fig. 1B), raising questions about the location of the protein in fungal cells (60). Besides the Ca²⁺-binding site, two histidine residues crucial for plant peroxygenase activity are present in the AFLA_00285 primary sequence, suggesting that this fungal protein might also act as an enzyme. However, compared to the sequences of Arabidopsis caleosins, both the N terminus and C terminus of AFLA_00285 are extended by additional motifs (Fig. 1A and C) that might impede substrate entrance and thus prevent peroxygenase activity.

AFLA_00285 is a peroxygenase. To test whether the fungal caleosin-like enzyme possesses peroxygenase activity, AFLA_00285 was expressed in yeast. As demonstrated for recombinant plant peroxygenases, crude extracts of yeast expressing AFA_00285 efficiently catalyzed the sulfoxidation of thiobenzamide (32.8 nmol min⁻¹ mg⁻¹ protein), the hydroxylation of aniline (24.6 nmol min⁻¹ mg⁻¹), and the epoxidation of oleic acid (36.2 nmol min⁻¹ mg⁻¹) in the presence of cumene hydroperoxide (Fig. 2A). No enzymatic activity was observed in the fractions isolated from yeast transformed with an empty vector (Fig. 2A).

When yeast crude extracts were subfractionated by differential centrifugations, only membrane fractions, i.e., microsomes and
lipid droplets, showed catalytic activity, while the supernatant fraction did not show activity (Fig. 2A). Since addition of the His tag to the N terminus or the C terminus of the protein did not modify its oxidative ability (not shown), we attempted to purify a His-tagged protein. Lipid droplet proteins were first solubilized (about 45%) with 0.2% Emulphogene, and then the solubilized enzymatic fraction (representing 85% of the total activity present in the lipid droplets) was purified by affinity chromatography on an Ni²⁺ column with a purification factor of 9.2 (Table 2). When analyzed by SDS-PAGE, the purified fraction showed one major single band at 32 kDa with a degree of purity higher than 98% when estimated by scanning densitometry (Fig. 2B). The purified fraction catalyzed the oxidation of thiobenzamide (1.55 μmol min⁻¹ mg⁻¹ of protein), aniline (1.24 μmol min⁻¹ mg⁻¹ of protein), and oleic acid (2.1 μmol min⁻¹ mg⁻¹ of protein) in the presence of cumene hydroperoxide as a cosubstrate. The heat-inactivated fraction was unable to catalyze such reactions. These data strongly suggest that peroxygenase activity is associated with the 32-kDa membrane-bound protein (Fig. 2C).

In Arabidopsis, such peroxygenase activity was assigned to a heme prosthetic group presumably bound to a histidine residue of the active site of the caelosin (30). The preservation of this amino acid in the primary structure of AFLA_002850 suggested that the fungal protein may also contain a heme group (Fig. 1A). Analysis of the light absorption spectra revealed a peak at 407 nm representative of the Soret band of hemoproteins. Addition of 1 mM calcium to the medium (Fig. 2G). Thus, calcium appears to be crucial for the structure and/or the activity of the A. flavus caelosin-like protein. Together these results favor a peroxygenase identity for AFLA_002850, and we have proposed to name this gene ApPXG (GenBank accession number KJ668859).

ApPXG efficiently metabolizes linoleic acid and its 13-hydroperoxide derivative. ApPXG catalyzed the oxidation of monounsaturated fatty acids (Fig. 3A). However, most of the oxylipins so far identified in pathogenic fungi derive from linoleic acid (24). To examine whether polyunsaturated fatty acids are also substrates, 14-C-labeled C₁₈ fatty acids with one to three double bonds were incubated with recombinant ApPXG in the presence of cumene hydroperoxide, H₂O₂, 9-HPOD, and 13–HPOD, and their metabolism was followed by TLC coupled with radiodetection. Linoleic acid was efficiently epoxidized by ApPXG followed by linolenic acid, whereas in comparison, only weak epoxidation of oleic acid was observed regardless of the hydroperoxide used. The most active cosubstrates were the fatty acid hydroperoxides followed by H₂O₂. In contrast, cumene hydroperoxide poorly promoted the epoxidation of unsaturated fatty acids (Fig. 3A).

Next, the metabolism of fatty acid hydroperoxides in the presence of the purified recombinant ApPXG was analyzed by HPLC (with UV detection). After 2 h of incubation, only 20% of the 9-HPOD and 42% of the 9-HPOD remained intact (peaks 1 and 2, respectively, in Fig. 3B and C, panels I). Peak 3 and peak 4 were identified to be the 13-HPOD, and their metabolization was followed by TLC with standards (Fig. 3Band C, panels II). No metabolites were detected from incubations of 9-HPOD and 13–HPOD with heat-inactivated ApPXG (Fig. 3B and C, panels III). These data indicate that recombinant ApPXG may preferably metabolize linoleic acid and its 13-hydroperoxide.

ApPXG is highly expressed in the early stage of spore germination. Plant caelosins/pxgs are present in seeds but also

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TABLE 2 Representative purification of oleosomal recombinant ApPXG³

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<th>Fraction</th>
<th>Vol (ml)</th>
<th>Total activity (nmol min⁻¹)</th>
<th>Amt of protein (mg [%])</th>
<th>Sp act (nmol min⁻¹ mg⁻¹)</th>
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<td>1.6 (33.3)</td>
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</table>

³ Recombinant ApPXG was solubilized in the presence of 0.2% Emulphogene for 45 min at 4°C. His-tagged ApPXG was purified by affinity chromatography on an Ni²⁺ column. The activity was measured with 1 mM aniline in the presence of 1 mM cumene hydroperoxide at 310 nm.
FIG 3 Peroxygenase activities of the purified recombinant AfPXG. (A) Co-oxidation of radiolabeled polyunsaturated fatty acids in the presence of cumene hydroperoxide (CuOOH), hydrogen peroxide (H₂O₂), 9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD), or 13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD). ¹⁴C-labeled substrates metabolized by purified recombinant AfPXG were separated by TLC and analyzed by radiodetection. C₁₈:₁, oleic acid; C₁₈:₂, linoleic acid; C₁₈:₃, linolenic acid. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Different lowercase letters indicate significant differences (P < 0.05) for the fatty acids used. (B and C) UV-HPLC analysis of the metabolization of fatty acid hydroperoxides by purified AfPXG. (I) Products formed after 2 h of incubation at 27°C of 13-HPOD or 9-HPOD by AfPXG; (II) 13-HPOD or 9-HPOD standards; (III) 13-HPOD or 9-HPOD incubated with heat-inactivated AfPXG. 9-HPOD and 13-HPOD did not differ in their ability to co-oxidize the reactions when the difference was analyzed by the t test. This FAOOH reduction capacity was significantly different from that measured in the presence of H₂O₂ and cumene hydroperoxide when analyzed by the t test.
in vegetative tissues, where they play distinct physiological roles. To examine whether \( AfPXG \) was constitutively expressed throughout the fungal life cycle or whether it was induced during particular phases of development, we evaluated \( AfPXG \) expression and \( AfPXG \) enzymatic activity in conidia, germinated spores, vegetative mycelium, and the conidiophore stage. The \( AfPXG \) transcription level was first evaluated by RT-PCR, and it level of expression was compared to that of the 18S rRNA gene, which was used as a reference. The accumulation of the \( AfPXG \) transcript was undetectable in the mature (nongerminated) conidia of \( A. \) flavus (time zero; Fig. 4A). The transcription of \( AfPXG \) was activated in the early stage of conidial germination (1 day after inoculation), and the level of the \( AfPXG \) transcript reached its maximal level after 2 days. The accumulation of \( AfPXG \) transcripts was reduced in the vegetative mycelium stage (days 4 and 5). Only low levels of \( AfPXG \) transcript were found in the conidiophore stage (days 6 and 7). Variations in the expression of \( AfPXG \) during development were confirmed by quantitative real-time RT-PCR analysis (Fig. 4B). \( AfPXG \) enzymatic activity was also assessed by measuring its capacity to oxidize the aniline of microsomal fractions isolated from the different developmental stages of \( A. \) flavus. Maximal peroxxygenase activity was measured (168.2 nmol min\(^{-1}\) mg\(^{-1}\) of protein) with microsomes isolated from germinated spores (day 2). Lower levels of enzymatic activity were found in microsomes isolated from vegetative mycelium and conidiophores (Fig. 4B). In summary, these results indicate that both the activation of \( AfPXG \) expression and the peroxxygenase activity of the \( AfPXG \)-encoded protein were maximal in the early stages of conidium germination of \( A. \) flavus.

The replacement of \( PXG \) by \( Hyg\) dramatically affects \( A. \) flavus development. Using fusion PCR, we deleted the \( PXG \)-like gene by creating DNA fragments carrying \( Hyg\) with \( \approx 1 \)-kb 5’ and 3’ flanking sequences from the \( A. \) flavus \( PXG \)-like gene. The fusion PCR products were introduced into the protoplast of \( A. \) flavus FSS63, and hygromycin-resistant colonies were isolated. The presence of the \( Hyg\) gene or the absence of the \( AfPXG \) gene in the genomic DNA of the transformant was confirmed by PCR (data not shown). In parallel, \( Hyg\) transformants were screened by Southern hybridizations, and approximately 65% of the transformants showed a pattern diagnostic for the replacement of \( PXG \) by \( Hyg\) (data not shown). We therefore use the abbreviation \( AfPXG\Delta \) to indicate this replacement. We examined the \( AfPXG\Delta \) strain for growth, sporulation, and aflatoxin production. The growth of the \( AfPXG\Delta \) strain was dramatically decreased, its biomass did not exceed 20% of that of the wild-type strain, and it failed to produce any remarkable spores after 7 days of culture. No expression of \( AfPXG \) was detected by qPCR, and no \( AfPXG \) activity was measured in the \( AfPXG\Delta \) strain. These results for biomass and sporulation did not change after 14 days of culture (data not shown). Due to the dramatic effects on fungal development caused by the deletion of \( AfPXG \), we next silenced \( AfPXG \) via a small interfering RNA (siRNA) approach to obtain putative intermediate phenotypes.

\( AfPXG \) expression is silenced by siRNA. Modifying the expression of a given gene is a powerful strategy to access biological functions. We therefore attempted to reduce \( AfPXG \) expression by the siRNA approach. The success of this strategy was evaluated by quantitative RT-PCR analysis and confirmed

![Image](http://aem.asm.org/ on June 21, 2017 by guest)
by measuring the enzymatic activity of AfPXG in the microsomal fractions isolated from strains in which AfPXG was silenced. The primers designed to be specific for the two siRNAs siRNAPXG1 and siRNAPXG2 differed in efficacy, with the primer specific for siRNAPXG1 being the most effective (Table 1). For example, compared to the results for the control strain, only 8% of the AfPXG transcript accumulated after the addition of 100 nM the primer specific for siRNAPXG1, whereas double that amount of gene expression was retained after the addition of the same concentration of the primer specific for siRNAPXG2 (Fig. 5A). Similarly, compared to the peroxynasenase activity in the control strain, peroxynasenase activities were more affected in the (+) siRNAPXG1 strain than in the (+) siRNAPXG2 strain (Fig. 5B). Thus, the siRNA strategy resulted in low levels of AfPXG peroxynasenase activity linked to the efficient reduction of AfPXG expression in the treated strains.

AfPXG silencing leads to fatty acid hydroperoxide accumulation. To examine whether AfPXG metabolized fatty acid hydroperoxides in the fungus like the recombinant protein did in vitro (see above), we first quantified the total hydroperoxide (ROOH) content present in the tissues of fungal strains in which AfPXG was silenced and the control strain. Hydroperoxide levels tripled and doubled in the (+) siRNAPXG1 and (+) siRNAPXG2 strains, respectively, compared to the level in the control (data not shown). The rise in the amount of ROOH was mostly due to the accumulation of fatty acid hydroperoxides, with the levels of 13-HPOD being higher than those of 9-HPOD in the two strains in which AfPXG was silenced (Fig. 6). Notably, the corresponding alcohol, 13-HOD, was present in WT A. flavus but was not be detected in the lines carrying siRNAPXG. These results are in agreement with a functional fatty acid hydroperoxide reductase activity supported by AfPXG in A. flavus.

Silencing of AfPXG reduces fungal biomass and leads to inhibition of conidial formation. The most striking visual effect of

![FIG 6 Repression of AfPXG expression resulted in hydroperoxide accumulation. The 9-HPOD and 13-HPOD contents in the tissues of the WT strain and strains in which AfPXG was silenced were analyzed. Each point represents the result of triplicate measurements. Values are means ± SDs (n = 3). The result for each line in which AfPXG was silenced was significantly different from that for the WT (*, P < 0.05; **, P < 0.01; ***, P < 0.001).](http://aem.asm.org/)

**TABLE 3** Dry weight of *A. flavus* mycelium in relation to AfPXG silencing before and after treatment with exogenous oxylipins

<table>
<thead>
<tr>
<th>A. flavus strain or strain and treatment</th>
<th>Mean ± SD mycelium wt (g DW per plate)a</th>
<th>CVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+) siRNAct</td>
<td>3.61 ± 0.034</td>
<td>0.96</td>
</tr>
<tr>
<td>(+) siRNAPXG1</td>
<td>1.13 ± 0.014</td>
<td>1.23</td>
</tr>
<tr>
<td>(+) siRNAPXG2</td>
<td>1.45 ± 0.026</td>
<td>1.81</td>
</tr>
<tr>
<td>Strain and treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT without oxylipins</td>
<td>3.73 ± 0.042</td>
<td>1.12</td>
</tr>
<tr>
<td>(+) siRNAPXG1 without oxylipins</td>
<td>1.15 ± 0.023</td>
<td>2.00</td>
</tr>
<tr>
<td>(+) siRNAPXG1 with 9-HOD</td>
<td>1.86 ± 0.024</td>
<td>1.29</td>
</tr>
<tr>
<td>(+) siRNAPXG1 with 13-HOD</td>
<td>2.07 ± 0.020</td>
<td>0.96</td>
</tr>
<tr>
<td>(+) siRNAPXG1 with mix-EOE</td>
<td>1.31 ± 0.032</td>
<td>2.44</td>
</tr>
<tr>
<td>(+) siRNAPXG1 with mix-HOE</td>
<td>1.24 ± 0.030</td>
<td>2.41</td>
</tr>
</tbody>
</table>

a Mean weight for fungal growth on six separate plates (n = 6) on day 7 after inoculation. DW, dry weight.
b CV, coefficient of variation, which was calculated as (standard deviation of the weight/mean weight) × 100.
c The mycelium weight for each silenced strain was significantly different from that for the WT (P < 0.05).
d The mycelium weight for treated strain (+) siRNAPXG1 was significantly different from that for the WT (P < 0.05).

![FIG 7 Phenotypes of strains in which AfPXG was silenced. (A) Phentotypes of the (+) siRNAPXG1 and (+) siRNAPXG2 strains compared with the phenotype of the (+) siRNAct control strain on PDA cultures. Cultures were carried out at 28°C for 7 days. (B) Evaluation of number of conidia and biomass in strains in which AfPXG was silenced and the control strain. The measurements were done in triplicate. Values are means ± SDs (n = 3). The number of conidia from each line in which AfPXG was silenced was significantly different from that for the WT when analyzed by the t test (*, P < 0.05; **, P < 0.01).](http://aem.asm.org/)
silencing of AfPXG was the poor fitness of the resulting strains compared to the fitness of the control. For example, only thin mycelia developed from the (+) siRNAPXG1 strain, with the mycelium weight representing about 30% of the mycelium weight of the control (Fig. 7A and B). Moreover, development of this strain was blocked at the reproductive stage because it failed to form conidia. The phenotypes were also attenuated in the (+) siRNAPXG2 strain. Its mycelial weight was still reduced compared to that of the control (it had about half of the mycelium weight of the control), but this strain was able to form conidia, although the conidia were 7-fold less numerous than they were in the control (Fig. 7B; Table 3). Thus, silencing of AfPXG resulted in a dramatic decrease of the growth of the transformed strains and a significantly reduced capacity to undergo conidiogenesis.

**Silencing of AfPXG leads to poor production of AFB1.** We next examined the effect of silencing of AfPXG on the production of aflatoxin B1 (AFB1). *A. flavus* FSS63 generated a unique peak coinciding with that of the AFB1 standard when analyzed by HPLC coupled with fluorescence detection (Fig. 8A, peak 2). By using a standard curve established with known concentrations of AFB1, about 13 μg ml⁻¹ of AFB1 was recovered from cultures of the control strain, siRNAc (Fig. 8A, inset). Silencing of AfPXG decreased the concentration of AFB1 by a factor of about 6 on day 7 after inoculation (Fig. 8A), suggesting a role of AfPXG in aflatoxin accumulation. The result for this one time point was confirmed by evaluating the time course of AFB1 production by the line in which AfPXG was silenced in comparison with that by the wild type (Fig. 8B).

To determine at what stage of the aflatoxin biosynthetic chain AfPXG was acting, we analyzed the expression of two crucial genes. The first one, aflD (alternatively named nor-1), intervenes in the early steps of aflatoxin biosynthesis when the first stable aflatoxin norsolorinic acid intermediate is converted into averantin. The second gene, aflR, is a regulatory gene involved in transcriptional activation of most of the structural genes. Expression of both genes was strongly repressed (by up to a factor of 51) in both strains in which AfPXG was silenced.
silenced and the control strain (Fig. 8C). Thus, silencing of \textit{AfPXG} contributes to a reduction in the expression of aflatoxin biosynthetic genes and, therefore, toxin accumulation.

\textbf{Silencing of \textit{AfPXG} enhances ROS-degrading enzyme activities.} Aflatoxin biosynthesis is stimulated under stress oxidative conditions that provoke reactive oxygen species (ROS) accumulation (61). To evaluate whether silencing of \textit{AfPXG} modified the fungal ROS status, we measured \textit{A. flavus} superoxide dismutase (SOD) and catalase (CAT) activities. These two enzymes control oxygen and \textit{H}_2\textit{O}_2 accumulation, respectively, in cells. SOD activity was stimulated by factors of 3 and 2 in strains (+) siRNA\textit{PXG}1 and (+) siRNA\textit{PXG}2, respectively, compared to its activity in the WT. CAT activity was similarly induced and doubled in the two strains, respectively, compared to its activity in the WT (Fig. 9). These data suggest that silencing of \textit{AfPXG} leads to enhanced ROS scavenging activities that probably result in reduced ROS accumulation in the transformed strains.

\textbf{Oxylipins derived from the \textit{AfPXG} pathway restore phenotypes in which \textit{AfPXG} is silenced.} To investigate whether phenotypes resulting from silencing of \textit{AfPXG} were specifically due to \textit{AfPXG} peroxygenase activity, we tried to complement strains in which \textit{AfPXG} was silenced chemically using oxylipins derived from \textit{AfPXG} metabolization of \textit{C}_{18:2} fatty acid hydroperoxides. Thus, the (+) siRNA\textit{PXG}1 strain was cultivated in the presence of 100 \textmu M 9-HOD, 13-HOD, mix-EOE, or mix-\textit{H}OE. The (+) siRNA\textit{PXG}1 strain was still able to colonize maize seeds. Compared to the levels achieved when they were complemented with 13-HOD, mix-EOE, and mix-\textit{H}OE were less effective (Fig. 10D and E). As a consequence, addition, conidiation was significantly reestablished (up to 60% compared to that in the wild type) in the strains in which \textit{AfPXG} was silenced if they were grown in the presence of these fatty acid alcohols but far less (about 20%) when they were grown in the presence of mixtures of epoxides or di- and trihydroxylated derivatives (Fig. 10C).

\textbf{Silencing of \textit{AfPXG} limits maize seed infection by \textit{A. flavus}.} Because the caleosin pathway present in \textit{Aspergillus flavus}. Similarly to plant caleosins, the enzymatically active \textit{AfPXG} was found to be associated with the endoplasmatic reticulum and lipid droplets (62), suggesting that this organelle might be a major site for oxylipin biosynthesis in fungi. However, the mode of anchorage of \textit{AfPXG} remains to be clarified because, like class II caleosins, it does not possess the canonical proline knot presumed to be crucial for targeting to lipid droplets (33).

\textbf{AfpXG activity is pivotal for fungal development.} \textit{Arabidopsis} possesses at least seven isofoms of caleosins (58). Each of them fulfills a distinct function linked to its catalytic activity (30, 33). In contrast, \textit{AfPXG} is a monogene copy in \textit{A. flavus} and might play multiple roles in fungal biology. Silencing of this gene severely affected and reduced fungal growth, conidium production, and aflatoxin accumulation. The restrained vegetative growth of the strains in which \textit{AfPXG} was silenced could partly result from a defect in spore germination, a developmental stage where the gene expression and enzymatic activity of the fungal caleosin were at their highest levels in wild-type \textit{A. flavus}. Because the caleosin \textit{ATS1} (63) was shown to control the germination of seeds by affecting the degradation of their lipid reserves (64), it can be postulated that \textit{AfPXG} might similarly promote spore germination by stimulating the metabolism of some spore reserve in the fungus.
The phenotypes of strains in which \textit{AfPXG} was silenced were due to peroxygenase activity. Addition of 100 \textmu M 9-HOD, 13-HOD, mix-EOE (mono- or diepoxides), or mix-HOE (di- or trihydroxy compounds) to the (+) siRNA\textit{PXG}1 strain restored fungal development. (A) Morphology and appearance of fungal growth on PDA medium; (B) mycelium dry weight; (C) conidium formation; (D and E) relative expression of \textit{aflD} (D) and \textit{aflR} (E) expression. (F) Aflatoxin B1 production. Controls (the WT strain and strains in which \textit{AfPXG} was silenced) were treated with ethanol. (G) The production of AFB1 in the treatments over time (2, 5, 7, 9, 11, and 13 days [d] after inoculation). Treatments and measurements were done in triplicate. Values are means ± SDs (\(n = 9\)). Asterisks indicate significant differences between treatment with each exogenous oxylipin and the control treatment (\(*, P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\)).
FAOHs were recently found to modulate reactive oxygen species phenotypes of strains in which AfPXG. Among them, FAOHs were more active in restoring the Aflox1 mutant resulted from the lack of production of oxylipins by AfPXG lines in which A. flavus (A) Maize seeds infected with a wild-type was correlated with a low cellular oxidative status under physiologically conditions. It is tempting to make a parallel with the fungal situation. We showed here that the alteration of AfPxG expression resulted in the accumulation of linoleic acid-derived hydroperoxides and to an increase in the activities of antioxidative enzymes, leading to low levels of ROS production. These data would explain, at least in part, the functioning of the fungal caleosin in aflatoxin production. Indeed, it was previously reported that 13-HPOD represses aflatoxin biosynthesis when added to Aspergillus cultures, whereas 9-HPOD lengthens the time during which aflatoxin transcripts accumulate (26). Thus, endogenous accumulation of both 13-HPOD and 9-HPOD in strains in which AfPxG was silenced might account for the poor accumulation of AFB1. On the other hand, the biosynthesis of aflatoxin is favored under conditions with high levels of ROS and silencing of AfPxG minimized ROS accumulation in fungal tissues. Thus, the combined effects of low levels of ROS and high levels of FAOOH production might account for the reduced production of aflatoxin in lines altered in AfPxG expression. This result would be in accordance with the findings of previous studies suggesting that the fungal cell responds to incomplete scavenging of reactive oxygen species at the intracellular level by producing toxins (61). Alternatively, repressed conidium and aflatoxin production in lines in which AfPxG was silenced might also result from the limited growth of the transformed fungus.

Aspergillus species produce aflatoxins at a time that coincides with spore development (66). In A. nidulans, these processes are regulated by G-protein signaling pathway components (67). Likewise, deletion of one β subunit of the heterotrimeric G-protein complex in A. flavus yielded an aconidial, aflatoxin-null phenotype for the resulting strain (66). Plant caleosin has been found to link to the G-protein component (68). Thus, at present, we cannot exclude the possibility that fungal caleosin and the oxylipins derived from it act by modulating the G-protein signaling pathway, resulting in an alteration of fungal development and, therefore, mycotoxin production.

Implication of AfPxG pathway in interactions between A. flavus and plant seeds. The incapacity of lines in which AfPxG was silenced to develop on maize seeds contrasts with the findings for Aflox1 strains, which, surprisingly, recovered the ability to form conidia and aflatoxin when inoculated onto viable corn kernels (65). It was thus presumed for the Aflox1 mutant that the oxylipins released by seeds induce the activation of secondary metabolic synthesis and fungal morphological changes (27, 65). Accordingly, it was earlier shown that the addition of 13-HPOD and 9-HPOD caused an increase in conidial development in A. flavus (24). Because the seed oxylipins closely resemble psi factors in structure, it was hypothesized that the sporogenetic effect of the seed oxylipins could take place through interference and/or mimicking of psi factors (66).

Our results, identifying a fungal caleosin using plant fatty acid hydroperoxides as the substrates, make the story more complex. Accordingly, the lack of recovery of conidiogenesis and aflatoxin production of the strains in which AfPxG was silenced in the presence of maize seeds might be due to their inability to generate active fungal FAOHs from the fatty acid hydroperoxides released by the maize seed lipoxygenase. This assumption would explain why conidiation and the production of aflatoxin were drastically reduced in Zmlox3 mutant fungi with a 9-LOX deletion infecting kernels (69). Similarly, this hypothesis would also explain how oxylipins generated by ZmLOX3 cloned in an aconidial ΔpooAC.
mutant of *A. nidulans* are able to restore the capacity of this mutant to produce conidia (27). Intriguingly, the growth of strains with AF*PXG* (Fig. 11) and AF*loxi* (65) was not rescued when they infected maize kernels. These results point to a specificity of fungal oxylipins for the control of *A. flavus* mass that act independently of the pathogenic process. In conclusion, by identifying a novel oxylipin enzyme, our data underline the importance of plant-like hydroxy fatty acid derivatives in fungal development and pathogenesis possibly through the control of oxidative status.

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