Seed Lipoxygenase Products Modulate *Aspergillus* Mycotoxin Biosynthesis

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Oilseed crops are frequently subject to contamination by mycotoxins produced by *Aspergillus* spp., particularly aflatoxin (AF) and to a lesser extent sterigmatocystin (ST). Several studies have suggested that metabolites generated from the plant lipoxygenase (LOX) pathway may either decrease or increase mycotoxin production by *Aspergillus* spp. We tested the possibility that the occurrence of seed LOX isozymes that produce distinct hydroperoxy fatty acids may account for these different effects on AF biosynthesis. For example, soybean LOX1 catalyzes the addition of O₂ to the C13 position of linoleic and linolenic acids while maize embryo LOX catalyzes the addition of O₂ to the C9 position. In vitro experiments showed that 13S-hydroperoxy fatty acids at concentrations of 10 and 100 µM repressed AF and ST pathway gene expression and significantly ($P = 0.05$) reduced AF and ST production in both *A. parasiticus* (AF producer) and *A. nidulans* (ST producer). Treatment with 1 µM 13S-hydroperoxy linoleic acid also significantly decreased AF production when introduced into growth media at continuous 24-h intervals. In contrast, the same concentrations of 9S-hydroperoxy linoleic acid did not reduce AF or ST production but extended the length of time AF and ST transcripts were detectable. These results show that 13S-hydroperoxy fatty acids directly or indirectly repress AF and ST biosynthesis and provide in vitro evidence that specific seed lipoxygenase activity could provide resistance to mycotoxin contamination by *Aspergillus* spp.

Additional keywords: aflatoxin B₁, 13S-hydroperoxy linolenic acid, methyl jasmonate, mRNA accumulation, plant-microbe interactions, *steU*, *ver-1*.

A continuing international problem in agriculture is the contamination of a number of oilseed crops with aflatoxin (AF) and to a lesser degree with sterigmatocystin (ST), two carcinogenic mycotoxins generated from the same biochemical pathway in several *Aspergillus* spp. (Diener et al. 1987; Holzapfel et al. 1966; Keller et al. 1995; Payne 1992). *Aspergilli* typically gain access to the seed through cracks generated by environmental stress (heat and/or drought) or via insect damage (Diener et al. 1987; Payne 1992). The most thorough

investigations of seed pathogenesis have been conducted with maize kernels, in which *Aspergillus* spp. preferentially colonize the lipid-rich embryo (Brown et al. 1993) and aleurone tissues (Keller et al. 1994a). Several studies have indicated a role for specific fungal degradative enzymes in *Aspergillus* pathogenesis (Brown et al. 1992; Cotty et al. 1990). Lipases seem to be important, since detailed histological work has shown that *A. flavus* first destroys the lipid bodies but not the starch granules of the maize scutellum (Smart et al. 1990). Generally, seed lipid bodies are primarily composed of palmitic (16:0), oleic (18:1), and linoleic (18:2) acids (Bewley and Black 1985), which support growth of *Aspergillus* spp. in vitro (Fanelli and Fabbri 1980). The polyunsaturated fatty acids (linoleic and linolenic, 18:3) are subject to both enzymatic and chemical oxidation (Gardner 1989). Considering that many in vitro studies have shown that lipid oxidation affects AF biosynthesis (Aziz et al. 1995; De Luca et al. 1995; Fabbri et al. 1983), we were interested in determining whether seed linoleic and linolenic acids are important components of the seed-*Aspergillus*-mycotoxin interaction.

Linoleic and linolenic acids are oxidized by the plant stress-response enzyme lipoxygenase (LOX) (Gardner 1995). LOX isozymes catalyze the formation of hydroperoxy fatty acids in a stereo-specific manner to yield 9S-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (9S-HPODE) and/or 13S-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13S-HPODE) from linoleic acid and 9S-hydroperoxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoic acid (9S-HPOTE) and/or 13S-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (13S-HPOTE) from linolenic acid (Gardner 1991). Some seed LOX primarily produce 13S-HPODE and 13S-HPOTE, e.g., soybean LOX-1 (Hamberg and Samuelsson 1967), and some produce primarily 9S-HPODE, e.g., maize embryo LOX (Gardner and Wiesleder 1970). 13S-HPODE and 13S-HPOTE can be further metabolized by plants to yield aldehydes with reported in vitro fungitoxic properties (Gardner 1991; Hildebrand 1989; Vaughn and Gardner 1993) and jasmonates, which are postulated to elicit production of plant defense proteins and secondary metabolites (Gardner 1991; Hildebrand 1989; Farmer and Ryan 1992). Metabolism of 9S-HPODE and 9S-HPOTE yields α - and γ -ketols, which have no known biological functions (Gardner 1991).

A possible relationship between plant LOX metabolites, *Aspergillus* growth, and AF and ST production is of interest because it suggests that a natural mechanism exists that

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affects AF and ST biosynthesis. Both inhibition and stimulation of AF by various LOX pathway metabolites have been reported. Methyl jasmonate (MJ) inhibited production of AF by *A. flavus* but not fungal growth on agar media (Goodrich-Tanrikulu et al. 1995), and aldehyde products of 13S-HPODE and 13S-HPOTE have been associated with the inhibition of germination of *A. flavus* spores and/or AF production (Doehle et al. 1993; Zeringue et al. 1996). This inhibition was positively correlated with linoleic acid levels in maize hybrids (Zeringue et al. 1996). However, a mixture of soybean LOX1 and linoleic acid (reported to produce an approximate 7:3 mixture of 13S-HPODE and 9S-HPODE) increased AF levels approximately 50 to 200 times in 10-day-old cultures of *A. parasiticus* and *A. flavus* (De Luca et al. 1995; Fabbri et al.

Table 1. Effects of different concentrations of linoleic acid (LA), 13S-HPODE, 13S-HPOTE, 9S-HPODE, and methyl jasmonate (MJ) on the dry weight of *Aspergillus parasiticus* strain ATCC 98016 mycelium

Treatment ^a	Mycelial weight ^b (mg)				
	24 h	48 h	72 h	96 h	96 cont. ^c
A&M + LA					
Control	4.61	5.64	5.40	4.42	6.55
1 μ M ^d	4.98	6.58	5.63	5.69	6.20
10 μ M	4.65	6.62	6.71	6.52	6.12
100 μ M	4.76	6.68	6.61	6.20	5.64
LSD _{0.05} ^e	ns	0.30	...	...	ns
A&M + 13S-HPODE					
Control	5.00	5.23	5.52	4.61	6.55
1 μ M	4.30	4.72	5.21	4.66	4.33
10 μ M	4.24	4.70	5.22	4.56	3.63
100 μ M	4.13	4.76	5.30	4.47	3.31
LSD _{0.05}	0.41	ns	...	...	1.04
A&M + 9S-HPODE					
Control	5.38	5.79	5.88	5.02	ND ^f
1 μ M	5.00	5.69	5.68	4.96	ND
10 μ M	5.17	5.65	5.71	5.08	ND
100 μ M	5.08	5.73	5.80	5.01	ND
LSD _{0.05}	ns	ns	...	...	...
A&M + 13S-HPOTE					
Control	5.05	5.42	ND	ND	ND
1 μ M	4.38	4.75	ND	ND	ND
10 μ M	4.21	4.59	ND	ND	ND
100 μ M	4.14	4.20	ND	ND	ND
LSD _{0.05}	0.54	0.61	...	...	...
A&M + MJ					
Control	5.45	5.87	ND	ND	ND
1 μ M	5.49	5.78	ND	ND	ND
10 μ M	5.51	5.82	ND	ND	ND
100 μ M	5.43	5.90	ND	ND	ND
LSD _{0.05}	ns	ns	...	...	...

^a A&M = aflatoxin-inducing medium (Adye and Mateles 1964) amended with 0.1% methanol. LA, 9S-HPODE, 13S-HPODE, 13S-HPOTE, and MJ were added to the A&M medium in the concentrations indicated.

^b Values are means of three replicates for the 24- and 48-h samples and two replicates for the 72- and 96-h samples. Values of continuous treatment of LA and 13S-HPODE (96 cont.) are means of three replicates.

^c LA and 13S-HPODE were added every 24 h in the concentrations indicated.

^d A&M was accidentally amended with 0.93, 9.3, and 93 μ M LA for the 24-, 48-, 72-, and 96-h experiments but with 1.0, 10.0, and 100 μ M LA for the 96 cont. treatment.

^e Means of triplicates were analyzed by using Fisher's protected least significant difference at $P = 0.05$; ns = not statistically significant.

^f Not determined.

1983; Passi et al. 1984). The mechanism or mechanisms by which these compounds affected AF and ST biosynthesis were not examined in these studies. Understanding these mechanisms and expressing the AF-inhibitory properties in susceptible plant species may lead to AF control strategies.

We hypothesized that LOX-specific placement of the hydroperoxy moiety on the ninth or thirteenth carbon atom of linoleic and linolenic acids could account for the differential effects on AF biosynthesis and that such effects would parallel changes in ST biosynthesis due to the functional conservation of the AF and ST gene cluster in *A. nidulans*, *A. flavus*, and *A. parasiticus* (Brown et al. 1996; Trail et al. 1995; Yu et al. 1995; Yu et al. 1996). To address this hypothesis, we examined the effects of purified 9S-HPODE, 13S-HPODE, and 13S-HPOTE on AF and ST gene expression and fungal growth of *A. parasiticus* and *A. nidulans* (AF producer and ST producer, respectively). Results of this study show that micromolar amounts of 13S-HPODE and 13S-HPOTE significantly inhibit accumulation of *ver-1* and *stcU* mRNA, AF and ST biosynthetic genes, respectively, and concomitant AF and ST production. In contrast, the same concentrations of 9S-HPODE lengthen the period of time that AF and ST mRNA pathway gene transcripts are detectable in culture. We propose that these two plant LOX products may act to differentially modulate AF and ST biosynthesis in oilseed crops.

RESULTS

Hydroperoxy fatty acids initially decrease growth of *Aspergillus* spp.

The effects of linoleic acid, 13S-HPODE, 9S-HPODE, 13S-HPOTE, and MJ on the growth of *A. parasiticus* are shown in Table 1. Addition of linoleic acid increased mean mycelial weight from 48 h and later time points. At 24 h, 100 μ M 13S-HPODE and 13S-HPOTE reduced mycelial growth, but the effect was not observed at later time points for 13S-HPODE. 9S-HPODE and MJ had no effect on the mycelial weight of *A. parasiticus*. When 13S-HPODE was added every 24 h during a total growth period of 96 h, mycelial weights were significantly reduced for all treatments (Table 1).

Both 13S-HPODE and 9S-HPODE but not linoleic acid decreased mycelial growth of *A. nidulans* (Table 2). Although the difference in weights was statistically significant only at concentrations of 10 and 100 μ M, mycelial weight reduction was observed for all treatments. As with *A. parasiticus*, the effects of these compounds on mycelial weight of *A. nidulans* declined by 48 h (Table 2).

13S-HPODE, 13S-HPOTE, and 9S-HPODE have different effects on accumulation of AF and ST mRNA.

ver-1 and *stcU*, homologous genes encoding a ketoreductase required for AF and ST biosynthesis in *A. parasiticus* and *A. nidulans*, respectively (Keller et al. 1994b; Skory et al. 1993), were used to evaluate accumulation of AF and ST pathway gene transcripts (Fig. 1). Three observations were readily apparent: 1) *ver-1* and *stcU* accumulations were similarly affected by hydroperoxy linoleic acids; 2) all concentrations of 13S-HPODE reduced transcript levels; and 3) all concentrations of 9S-HPODE extended the period of time transcript could be detected.

Addition of linoleic acid increased *ver-1* transcript levels at

48 and 72 h compared with the control but did not alter the timing of expression (Fig. 1A). There was little effect of this fatty acid on *stcU* transcript, which was strongly expressed in both control and linoleic acid treatments at 24 and 48 h. *stcU* mRNA declined at 72 h, and there was little or no accumulation at 96 h. In contrast, the hydroperoxy products of linoleic acid differentially affected levels of both transcripts.

The addition of 13S-HPODE to the medium reduced *ver-1* and *stcU* levels in a concentration-dependent manner (Fig. 1B). Treatment with 100 μ M appeared to delay the expression of mRNA (Fig. 1B), but the sensitivity of detection was not powerful enough to distinguish between reduction and delay of transcript. As in the control, transcripts could no longer be detected at 96 h in 13S-HPODE treatments. A similar trend was observed in *A. parasiticus* cultures grown in medium amended with 13S-HPODE; that is, the treatments with 1 and

10 μ M had less effect and the treatment with 100 μ M a greater effect on *ver-1* accumulation (e.g., the transcript could not be detected in the treatment with 100 μ M at any time point) (data not shown).

Unlike 13S-HPODE, 9S-HPODE had little effect on *ver-1* and *stcU* transcript accumulation patterns at 24 and 48 h; however, by 72 h, transcript levels appeared slightly higher in 9S-HPODE treatments compared with the controls (Fig. 1C). At 96 h, when *ver-1* could no longer be detected in the control, linoleic acid, or 13S-HPODE treatments, all concentrations of 9S-HPODE resulted in *ver-1* expression (Fig. 1C). *stcU* transcript levels were also higher than those of the control at 72 and 96 h (Fig. 1C).

Different experiments yielded some variation in the intensity of *ver-1* and *stcU* signals in the control medium, but the described effects of hydroperoxy fatty acids were consistent throughout the study.

Table 2. Effects of different concentrations of linoleic acid (LA), 13S-HPODE, and 9S-HPODE on the dry weight of *Aspergillus nidulans* strain FGSC26 mycelium

Treatment ^a	Mycelial weight ^b (mg)	
	24 h	48 h
CAM + LA		
Control	10.23	11.62
1 μ M	10.53	11.57
10 μ M	10.34	12.04
100 μ M	9.94	11.56
LSD _{0.05} ^c	ns	ns
CAM + 13S-HPODE		
Control	6.67	12.50
1 μ M	5.30	12.00
10 μ M	4.23	11.40
100 μ M	3.80	11.00
LSD _{0.05}	0.69	ns
CAM + 9S-HPODE		
Control	10.62	12.20
1 μ M	9.42	11.55
10 μ M	8.32	11.25
100 μ M	7.65	10.95
LSD _{0.05}	1.29	ns

^a CAM (control) = complete ammonium medium (sterigmatocystin inducing) amended with 0.1% methanol. LA, 9S-HPODE, and 13S-HPODE were added to CAM in the concentrations indicated.

^b Values are means of three replicates.

^c Means were analyzed by using Fisher's protected least significant difference at $P = 0.05$; ns = not statistically significant.

13S-hydroperoxy fatty acids reduce mycotoxin production.

In general, transcript expression preceded mycotoxin synthesis by 3 to 12 h.

Detection of aflatoxin B₁ (AFB₁) by enzyme-linked immunosorbent assay (ELISA). Figure 2 depicts the effects of linoleic acid, 13S-HPODE, and 9S-HPODE at concentrations of 1 to 100 μ M on AFB₁ levels in *A. parasiticus*. AFB₁ levels were significantly reduced in cultures treated with 13S-HPODE at concentrations of 10 and 100 μ M from 24 to 96 h compared with all other treatments ($P = 0.05$). At 24 h, linoleic acid (100 μ M) and 9S-HPODE significantly reduced AFB₁ production (Fig. 2C), but a significant effect was not observed from 48 to 96 h (Fig. 2C and Table 3).

Continuous treatment of *A. parasiticus* with 13S-HPODE significantly reduced AFB₁ production at all concentrations tested compared with linoleic acid and the control (Fig. 3). In this instance, the treatment with 1 μ M 13S-HPODE appeared as effective as the treatments with 10 and 100 μ M.

High-performance liquid chromatography (HPLC) detection of AFB₁ and ST and spectrophotometric detection of norsolorinic acid. Table 3 summarizes the results from additional experiments on the effects of 13S-HPODE, 9S-HPODE, and linoleic acid on AFB₁ production by *A. parasiticus*. Effects of MJ and 13S-HPODE on AFB₁ and norsolorinic acid (NOR) and 13S-HPODE on ST production by *A. nidulans* were also analyzed (Table 3). Increasing concentrations of 13S-HPODE resulted in decreasing levels of AF and ST production by *A.*

Fig. 1. Effects of 1, 10, and 100 μ M linoleic acid, 13S-HPODE, and 9S-HPODE on *Aspergillus parasiticus ver-1* and *A. nidulans stcU* accumulation at 24, 48, 72, and 96 h. *A. parasiticus* RNA (20 μ g) was hybridized with *ver-1* DNA (top), and 20 μ g of *A. nidulans* RNA was hybridized with *stcU* DNA (bottom). rRNA stained with ethidium bromide is shown below transcripts. 1 = 1 μ M; 10 = 10 μ M; 100 = 100 μ M; and C = control media with no additives.

Table 3. Effects of three different concentrations of linoleic acid (LA), 13S-HPODE, 13S-HPOTE, 9S-HPODE, and methyl jasmonate (MJ) on mycotoxin production by *Aspergillus parasiticus* strain ATCC 98016 and *A. nidulans* strain FGSC26

Fungus Treatment ^a	AFB ₁ and NOR levels ^b (µg/ml)			
	24 h	48 h	72 h	96 h
<i>A. parasiticus</i> ATCC 98016				
A&M + LA				
Control	0.20	0.47	0.29	0.33
0.93 µM	0.26	0.43	0.26	0.31
9.3 µM	0.20	0.54	0.54	0.54
93 µM	0.26	0.45	0.58	0.39
A&M + 9S-HPODE				
Control	0.33	0.33	1.04	0.84
1.0 µM	0.22	0.19	0.99	0.64
10 µM	0.26	0.34	0.65	0.44
100 µM	0.28	0.51	0.53	0.56
A&M + 13S-HPODE				
Control	0.70	2.09	1.53	1.15
1.0 µM	0.33	0.33	1.05	0.74
10 µM	0.15	0.20	1.08	0.75
100 µM	0.04	0.04	0.56	0.67
A&M + 13S-HPOTE				
Control	0.45 (9.85)	0.68 (17.48)	ND ^c	ND
1.0 µM	0.39 (7.40)	0.74 (15.53)	ND	ND
10 µM	0.48 (3.93)	0.78 (16.19)	ND	ND
100 µM	0.00 (0.01)	0.00 (1.93)	ND	ND
A&M + MJ				
Control	0.14 (0.71)	0.31 (5.70)	ND	ND
1.0 µM	0.14 (1.14)	0.35 (4.87)	ND	ND
10 µM	0.14 (1.23)	0.31 (4.46)	ND	ND
100 µM	0.16 (1.56)	0.21 (4.08)	ND	ND
<i>A. nidulans</i> FGSC26				
CAM + 13S-HPODE				
Control	1.25	4.17	ND	ND
1.0 µM	0.00	1.98	ND	ND
10 µM	0.00	1.44	ND	ND
100 µM	0.00	0.81	ND	ND

^a A&M = aflatoxin-inducing medium (Adye and Mateles 1964) amended with 0.1% methanol. CAM (control) = complete ammonium medium (sterigmatocystin inducing) amended with 0.1% methanol. LA, 9S-HPODE, 13S-HPODE, 13S-HPOTE, and MJ were added in the concentrations indicated.

^b Aflatoxin B₁ (AFB₁) and sterigmatocystin (ST) were measured by high-performance liquid chromatography. Norsolorinic acid (NOR) was measured by spectrophotometry. NOR values are presented in parentheses after AFB₁ values in the 13S-HPOTE and MJ treatments. Values are averages of three replicates.

^c Not determined.

Fig. 2. Effects of 1, 10, and 100 µM linoleic acid, 13S-HPODE, and 9S-HPODE on AFB₁ production by *Aspergillus parasiticus* at 24, 48, 72, and 96 h. AFB₁ levels are presented as means of three replicates. Bars indicating standard deviation were calculated from results of analysis of variance. Different treatments are indicated by different bar shades.

parasiticus and *A. nidulans*, respectively. ST could not be detected in any of the 24-h 13S-HPODE treatments; and at 48 h, ST levels were decreased by 13S-HPODE in a concentration-dependent manner.

Concentrations of 1 and 10 µM 13S-HPOTE had little effect on AFB₁ but at 24 h did reduce levels of the AF and ST pathway intermediate, NOR (NOR is produced at ~10-fold greater levels than AFB₁ in *A. parasiticus* strain ATCC 98016 and is thus useful for measuring low mycotoxin levels) (Keller et al. 1994a). As was predicted by the transcript data described above, no AFB₁ and very little NOR were detected in the treatment with 100 µM 13S-HPOTE at 24 or 48 h (Table 3). In contrast to the negative correlation between mycotoxin levels and 13S-hydroperoxy concentrations, there was no consistent correlation between 9S-HPODE or MJ concentration and AFB₁ (Table 3) or ST production (data from thin-layer chromatography [TLC] not shown).

In prior studies, researchers reported that AF production increased approximately 50 to 200 times after *A. parasiticus* cultures were treated for 10 days with a mixture of 1 mM linoleic acid and soybean LOX-1 (presumed to produce a 7:3 ratio of 13S-HPODE to 9S-HPODE) (De Luca et al. 1995; Fabbri et al. 1983; Passi et al. 1984). An experiment was set up to determine whether this increase in AF production could be caused by differential effects of 9S-HPODE or 13S-HPODE. Ten days' growth of *A. parasiticus* in a control medium, 1 mM linoleic acid, 1 mM 9S-HPODE, or 1 mM linoleic acid plus soybean LOX-1 yielded low levels of AFB₁ (range, 0.01 to

Fig. 3. Effects of addition of 1, 10, and 100 µM 13S-HPODE to growth media every 24 h up to 96 h on AFB₁ production by *Aspergillus parasiticus*. AFB₁ levels are presented as means of three replicates. Bars indicating standard deviation were calculated from results of analysis of variance. Different treatments are indicated by different bar shades.

0.1 µg/ml; mean, ~0.04 µg/ml). These values were not significantly different from each other and were 10 times lower than AFB₁ levels recorded at earlier time points (Fig. 2 and Table 3). AF could not be detected in treatments with 1 mM 13S-HPODE, although slight amounts of NOR were visualized by TLC (data not shown).

To assess the effects of mixtures of hydroperoxy linoleic acids on NOR and AF production, *A. parasiticus* cultures were treated with 10 µM 9S-HPODE, 13S-HPODE, or various mixtures (9:1, 1:1, and 1:9 [vol/vol]) of these two compounds. Aside from the decrease in AF production in the pure 13S-HPODE treatment, there was little difference between treatments, although the total amount of NOR plus AF was highest in the mixed treatments (Fig. 4).

DISCUSSION

Secondary metabolism qualifies as one of the least understood processes of fungal biology. This is reflected in the continuing inability to significantly reduce mycotoxin production in infected crops. By gaining an understanding of the molecular mechanisms leading to the expression of AF and ST pathway genes, it should be possible to design logical AF and ST control strategies. For example, recent molecular studies of the aspergilli have clearly established that the genes involved in ST and AF biosynthesis are functionally conserved within an ~60-kb gene cluster in *A. nidulans*, *A. flavus*, and *A. parasiticus* (Brown et al. 1996; Trail et al. 1995; Yu et al. 1995). The expression of these cluster genes is dependent on *afR*, a pathway-specific transcription factor whose expression is also regulated during the life cycle of the fungus (Chang et al. 1995; Woloshuk et al. 1994; Yu et al. 1996). It appears that some aspects of fungal morphological differentiation are tightly correlated with AF and ST biosynthesis, since *Aspergillus* developmental mutants are often atoxigenic (Kale et al. 1996; Keller et al. 1995). The identification of plant metabolites that alter expression of AF or ST genes and/or fungal development may contribute to the elimination of mycotoxin contamination of agricultural commodities. Results

presented here suggest that plant lipoxygenases could be candidate molecules for such strategies.

The plant LOX pathway produces metabolites implicated in pathogen resistance (for reviews, see Gardner 1991 and 1995, Hildebrand 1989, and references therein). This pathway is activated by abiotic and biotic stress factors including pathogen attack (Melan et al. 1993; Peng et al. 1994). The ensuing LOX-derived metabolites are reported to affect growth and development in many plant-pathogenic fungi (Masui and Kojima 1990; Ohta et al. 1990; Ricker and Bostock 1993; Vaughn and Gardner 1993). Several studies have presented data showing that LOX pathway metabolites either stimulate (De Luca et al. 1995; Fabbri et al. 1983; Passi et al. 1984) or inhibit (Doehlert et al. 1993; Goodrich-Tanrikulu et al. 1995; Zeringue et al. 1996) AF production in *Aspergillus* spp. Because different seed LOX isozymes primarily produce either 9S- or 13S-hydroperoxy fatty acids, it seemed reasonable to explore the possibility that this specificity accounted for the variations in AF production described above.

Purified 13S-HPODE and 9S-HPODE clearly had differential effects on AF and ST gene transcription (Fig. 1). Specifically, micromolar concentrations of 13S-HPODE and 13S-HPOTE suppressed *stcU* and *ver-1* mRNA accumulation (Fig. 1B and data not shown). The most obvious effect of 9S-HPODE was to extend the age of the culture from which *ver-1* and *stcU* mRNA could be detected (Fig. 1C). AFB₁ levels reflected the pattern of *ver-1* transcript accumulation (Figs. 1 and 2); a similar correlation was observed between ST levels and *stcU* accumulation (Fig. 1 and Table 3). Most importantly, 13S-HPODE and 13S-HPOTE consistently reduced AFB₁ levels (Figs. 1 and 2 and Table 3). This reduction was statistically significant at $P = 0.05$ for 10 and 100 µM 13S-HPODE treatments of *A. parasiticus* (Fig. 2). Addition of 1 µM 13S-HPODE every 24 h also resulted in a significant reduction in AFB₁ (Fig. 3). These results show that 13S-HPODE and 13S-HPOTE, directly or indirectly, inhibit the expression of AF and ST pathway genes.

MJ, a derivative of 13S-HPOTE, did not suppress AFB₁ levels or *ver-1* mRNA in this study. This is in contrast to the AF inhibitory effects of MJ on an *A. flavus* strain (Goodrich-Tanrikulu et al. 1995). Further evaluations of MJ's effects on mycotoxin production in different *Aspergillus* strains are needed. Our results also did not support earlier studies describing hydroperoxy fatty acid stimulation of AF production in *A. flavus* and *A. parasiticus* (De Luca et al. 1995; Fabbri et al. 1983; Passi et al. 1984). We found that after 10 days, AFB₁ production was not differentially affected by linoleic acid, a LOX1-linoleic acid mixture, or 9S-HPODE. The only notable observation was the lack of detectable AFB₁ in 1 mM 13S-HPODE cultures. In fact, all 10-day treatments produced levels of AFB₁ that were approximately 10 times lower than those produced at earlier time points (24 to 96 h). Although there were modest increases in AFB₁ and/or NOR levels in some 9S-HPODE treatments (Fig. 2C and Table 3) and 13S- and 9S-HPODE mixtures (Fig. 4), they were not enough to conclude that 9S-HPODE stimulated AF biosynthesis. Still, the reports of lipoperoxide stimulation of AF (Aziz et al. 1995; De Luca et al. 1995; Fabbri et al. 1983; Fanelli et al. 1983) coupled with the effects of 9S-HPODE on AF and ST gene accumulation (Fig. 1C) are perplexing and not easy to dismiss. The significance of 9S-HPODE-associated *ver-1* and *stcU* expression is being examined.

Fig. 4. Effects of mixtures of 10 µM 13S-HPODE plus 9S-HPODE (vol/vol) on AFB₁ and norsolorinic acid (NOR) production by *Aspergillus parasiticus*. Totals of AFB₁ (AF) and NOR produced by *A. parasiticus* are given at the indicated time points. Extract from the 96-h 13S-HPODE treatment was slightly contaminated with extract from the 96-h 9S-HPODE plus 13S-HPODE (1:9) treatment. Different treatments are indicated by different bar shades.

A possible explanation of how 13S-HPODE represses AF and ST biosynthesis may be that it affects fungal developmental processes required for normal AF and ST biosynthesis. Lipoxygenase activity has been reported in fungi (Shechter and Grossman 1983), and at least two species, *Ceratocystis ulmi* and *Lagenidium giganteum*, are altered in morphological development when grown in the presence of LOX inhibitors (Jensen et al. 1992; Kerwin et al. 1986). Of interest is the finding that *A. nidulans* has endogenous LOX activity responsible for the production of a pheromone (composed of a mixture of hydroxy linoleic acids) necessary for sexual development (Champe and El-Zayat 1989; Mazur et al. 1991). Preliminary data suggests that soybean LOX-1 expression interferes with sexual development in *A. nidulans* (A. M. Calvo, T. M. Sanford, and N. P. Keller, unpublished data). Further studies will be aimed at elucidating a possible signaling role of oxidized fatty acids on the development of *Aspergillus* spp. and subsequent AF and ST production.

In summary, we have demonstrated that the two primary products of LOX, 13S- and 9S-hydroperoxy fatty acids, alter AF and ST pathway gene expression and mycotoxin production in *Aspergillus* spp. These results provide further evidence that soybean LOX-1 could be involved in soybean's notable resistance to infection by *Aspergillus* (Doehlert et al. 1993) and suggest that 13S-specific lipoxygenases could be utilized in an in situ strategy to control AF levels in susceptible crops.

MATERIALS AND METHODS

Fungal stocks and chemicals.

Fungal isolates used in this study were the ST producer *A. nidulans* FGSC26 (*ve-1*, *biA*) and the AF producer *A. parasiticus* ATCC 98106 (*wh*, *nor-1*) (Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, and American Type Culture Collection, Rockville, MD, respectively). *A. parasiticus* ATCC 98106 is an AF mutant that accumulates both AF and NOR, a precursor in the AF and ST pathway (Keller et al. 1994a). The fact that this strain produces higher levels of NOR than of AF allows for NOR quantitation under conditions of low AF biosynthesis.

Both isolates were maintained as silica (room temperature) or glycerol (−80°C) stocks. *A. parasiticus* was grown at 30°C on potato dextrose agar and *A. nidulans* at 37°C on *Aspergillus* minimal nitrate medium agar (Cove 1966) with appropriate supplements (e.g., biotin) for conidial production. Conidia were harvested with an aqueous solution of 0.1% (vol/vol) Tween 20 and adjusted to the desired inoculum concentration.

AF, ST, soybean lipoxygenase-1 (LOX-1, type 1S), linoleic and linolenic acids, and reagents for ELISA were obtained from Sigma (St. Louis, MO). NOR standards were previously prepared in our laboratory after NOR was purified from extracts of *A. parasiticus* ATCC 98106. MJ was provided by E. Bell and J. Mullet (Texas A&M University).

Synthesis of hydroperoxy fatty acids from linoleic and linolenic acids.

13S-HPODE and 13S-HPOTE were prepared by oxidizing linoleic or linolenic acid, respectively, with soybean LOX-1. A 3-mM mixture of the appropriate fatty acid was prepared in 100 ml of distilled water and 0.4% (vol/vol) Tween 20. The sample was emulsified by sonication, and the pH was adjusted

to 10 with 1 N KOH. Also, a LOX-1 solution (0.2 mg/ml) was prepared in 360 ml of distilled water and 40 ml of 0.1 M potassium borate buffer, which was adjusted to pH 10 with 1 N KOH. The two solutions were cooled to 10°C, combined, and subjected to oxygenation by bubbling pure oxygen through the mixture for 30 to 40 min at 21°C, at which time the mixture was adjusted to pH 4 with 5 M HCl and extracted with chloroform and methanol (2:1 [vol/vol]). The chloroform fractions containing the reaction products were evaporated to dryness in a rotary evaporator, redissolved in ethyl ether, and purified on a SilicAR CC-4 column (Mallinckrodt, St. Louis, MO) by elution with increasing concentrations of acetone in hexane as previously described (Gardner and Newton 1987). The purity of the product obtained was verified by reverse-phase HPLC and comparison to a pure sample obtained from BIOMOL (Plymouth Meeting, PA).

The 9S-HPODE was prepared from a crude extract of ripe tomato fruit (~250 g) in a manner similar to the procedure described by Matthew et al. (1977). Briefly, ripe tomatoes (Roma) were peeled, the seeds discarded, and the pulp homogenized for 1 min in 150 ml of cold, 0.5 M NaOAc buffer (pH 5.5). Ammonium linoleate, prepared by adding 2 mM linoleic acid to 1 N NH₄OH, was added to the pulp, making a final concentration of 1 mM. The resulting mixture was then homogenized and incubated for approximately 40 min at 25°C and further processed as described for the synthesis of 13S-HPODE and 13S-HPOTE. The 9S-HPODE product was compared with the peak obtained from a pure sample obtained from BIOMOL. All three hydroperoxy products (13S-HPOTE, 13S-HPODE, and 9S-HPODE) were prepared as solutions in methanol. Concentrations of the fatty acid hydroperoxides were determined spectrophotometrically from absorbance values at 234 nm (Gardner and Newton 1987). Several samples were further analyzed by reactions with triphenylphosphine followed by HPLC measurements to confirm purity (courtesy H. Gardner).

Fungal growth conditions.

Mycelial cultures of *A. parasiticus* inoculated with 1×10^6 spores per milliliter were grown for 36 h at 250 rpm at 30°C in 500 ml of peptone mineral salt formulation, an AF noninducing medium (Buchanan et al. 1984). Mycelia were harvested by suction filtration through Miracloth (Calbiochem, La Jolla, CA). Equal fresh weights of mycelia (~400 mg wet weight) were then transferred into 40 ml of the AF-inducing medium A&M (Adye and Mateles 1964). Single-component treatments were A&M medium amended at the time of shift with 1, 10, or 100 µM of linoleic acid, 13S-HPODE, 13S-HPOTE, or 9S-HPODE for a total of 12 treatments. Control cultures were grown in A&M medium containing 0.1% methanol (the solvent for the fatty acids). Two-component treatments consisted of mixtures of 10 µM 9S-HPODE and 10 µM 13S-HPODE at ratios of 9:1, 1:1, and 1:9 (vol/vol). Control cultures were grown on A&M medium amended with 10 µM 9S-HPODE or 13S-HPODE only.

The effects of the continual addition of 13S-HPODE were tested by adding 1, 10, or 100 µM linoleic acid and 13S-HPODE at time of transfer and every 24 h until harvest (96 h). Control cultures were grown in A&M medium supplied with continual addition of 0.1% methanol.

Finally, in efforts to repeat the experiments in which 100-fold increases in AF were reported (De Luca et al. 1995; Fabbri et al.

1983; Passi et al. 1984), *A. parasiticus* cultures were grown for 10 days in 100 or 1,000 μ M 13S-HPODE, 9S-HPODE, linoleic acid, or mixtures of soybean LOX-1 and linoleic acid.

Mycelium and culture filtrates were harvested at 24, 48, 72, or 96 h or 10 days after transfer. Time points were replicated two to four times, with exception of the 9S- plus 13S-HPODE mixture experiment. Mycelial dry weights were obtained from three replicates to analyze effects on growth. Filtrates from three replicates were analyzed for AF production. A subset of replicate sample was analyzed for NOR production. Mycelia from at least two replicates were saved for mRNA analysis.

Mycelial cultures of *A. nidulans* were started by inoculating complete nitrate medium (minimal medium with addition of 0.1% yeast extract, 0.1% casein hydrolysate, 0.2% peptone, and another 10 g of glucose per liter) with 1×10^6 spores per milliliter and growing the culture at 300 rpm, 37°C, for 12 h. By the procedure described above, mycelia were transferred to 40 ml of the ST-inducing medium, complete ammonium media (CAM, in which ammonium tartrate rather than nitrate was used as the nitrogen source). Treatments were CAM amended at the time of shift with 1, 10, or 100 μ M of linoleic acid, 13S-HPODE, or 9S-HPODE for a total of nine treatments. Control cultures were grown in CAM containing 0.1% methanol. Subsequent procedures were similar to those applied to *A. parasiticus*.

mRNA expression.

Mycelial samples used for RNA analysis were harvested by suction filtration through Miracloth, frozen in liquid nitrogen, and stored at -80°C until isolation. Total RNA was isolated from mycelia by using Trizol as prescribed by the manufacturer (GIBCO BRL, Gaithersburg, MD). Approximately 20 μ g of total RNA per sample was separated by electrophoresis in 1.2% denaturing formaldehyde agarose gel and then transferred to Hybond nylon membrane (Amersham, Arlington Heights, IL) by capillary action. Equal loading of RNA was determined by ethidium bromide staining of rRNA.

ver-1 and *stcU*, homologous genes encoding a ketoreductase essential for AF and ST production in *A. parasiticus* (Skory et al. 1993) and *A. nidulans* (Keller et al. 1994b), respectively, were used to analyze AF and ST gene expression. These genes consistently produce strong signals and are representative of expression of other enzymatic genes in the AF and ST gene clusters (Brown et al. 1996; Chang et al. 1995; Skory et al. 1993). Expression of *ver-1* was determined by probing with a 0.50-kb *Bam*HI-*Eco*RI fragment of pBSV2 (Skory et al. 1993). Expression of *stcU* (formerly referred to as *verA*) (Keller et al. 1994b) in *A. nidulans* was analyzed by probing with a 0.70-kb *Ksp*I-*Sma*I fragment from pRB7 (Yu et al. 1996). Probes were prepared by random priming procedures (Sambrook et al. 1989) with (^{32}P)dCTP. mRNA analyses of each treatment were repeated two to four times. Each time period (e.g., 24, 48, 72, or 96 h) was an experimental unit, and, as expected, some variation resulting from technical processes was observed between experiments in the intensity of *ver-1* and *stcU* signals in the control medium.

Quantification of mycotoxins.

The common practice of measuring AFB₁ rather than all four AFs (AFB₁, AFG₁, AFB₂, and AFG₂) was followed throughout this study. AFB₁ is produced in higher amounts than any other of the AFs (Keller et al. 1994a).

ELISA detection of AFB₁. AFB₁ was analyzed from the filtrate by indirect competitive ELISA as described previously (Pestka 1988).

HPLC detection of AFB₁. AFB₁ was also extracted from 10 ml of *A. parasiticus* culture medium by a chloroform-acetone procedure and quantitated by HPLC as previously described (Keller et al. 1994a). All HPLC data are presented as the average of three replicates per treatment.

Spectrophotometric detection of NOR. For a select subset of experiments, NOR was extracted from the same culture filtrate used for AF analysis. Culture filtrate extract (10 ml) was evaporated to dryness and resuspended in 1 ml of acetone. The acetone sample was divided into two aliquots, 0.5 ml for AF analysis and 0.5 ml for NOR analysis. The NOR aliquot was evaporated to dryness and then resuspended in 0.5 ml of methanol plus 1 N sodium hydroxide (90:10 [vol/vol]), pH $\sim$ 10.0. The resulting crude extract was diluted 10- to 50-fold in the same solution depending on color intensity, and a 200- μ l aliquot was measured on a 96-well titer ELISA plate reader at 595 nm. This data was normalized to a NOR standard.

HPLC detection of ST. ST was extracted and analyzed from lyophilized mycelia of *A. nidulans*. Briefly, lyophilized mycelia were crushed with a spatula to fine powder and resuspended in 10 ml of chloroform plus acetone (1:1 [vol/vol]) for 30 min with gentle agitation. The resulting slurry was centrifuged at 5,000 rpm for 10 min; the supernatant was collected; and the organic solvent was evaporated to dryness under the fume hood. The crystals were dissolved in 0.5 ml of chloroform before HPLC. Methods used for HPLC were described previously (Keller et al. 1994a). All HPLC data are presented as the average of three replicates per treatment. Alternatively, ST was assessed by visualization on TLC (Keller et al. 1994b).

Statistical analysis.

Data were analyzed by two-way analysis of variance. Differences between means were evaluated by Fisher's protected least significant difference and depicted by standard deviation bars for figures.

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