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# Autoxidated linolenic acid inhibits aflatoxin biosynthesis in *Aspergillus flavus* via oxylipin species

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ABSTRACT

Aflatoxins produced by *Aspergillus* species are among the most toxic and carcinogenic compounds in nature. Although it has been known for a long time that seeds with high oil content are more susceptible to aflatoxin contamination, the role of fatty acids in aflatoxin biosynthesis remains controversial. Here we demonstrate in *A. flavus* that both the saturated stearic acid (C18:0) and the polyunsaturated linolenic acid (C18:3) promoted aflatoxin production, while C18:3, but not C18:0, inhibited aflatoxin biosynthesis after exposure to air for several hours. Further experiments showed that autoxidated C18:3 promoted mycelial growth, sporulation, and kojic acid production, but inhibited the expression of genes in the AF biosynthetic gene cluster. Mass spectrometry analyses of autoxidated C18:3 fractions that were able to inhibit aflatoxin biosynthesis led to the identification of multiple oxylipin species. These results may help to clarify the role of fatty acids in aflatoxin biosynthesis, and may explain why controversial results have been obtained for fatty acids in the past.



## 1. Introduction

Aflatoxins (AFs) are a group of highly toxic and carcinogenic natural compounds produced by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* in seeds, both pre- and post-harvest. These compounds pose serious threats to human and animal health (Amaike and Keller, 2011; Kensler et al., 2011; Roze et al., 2013). It has been known for a long time that seeds of plants such as maize, peanut, and walnut with high oil contents are more susceptible to AF production following infection by *Aspergillus* species (Fabbri et al., 1980; Reddy et al., 1992; Severns et al., 2003). It is also known that unprocessed seeds support AF production better than defatted seeds (Reddy et al., 1992; Mellon et al., 2000). Studies in maize have shown that AF accumulation is more abundant in oil-rich embryos and aleurone layers as compared to starchy endosperms (Brown et al., 1993; Keller et al., 1994). These results suggest that fatty acids (FA) are important for AF biosynthesis. However, *in vitro* experiments show that stearic acid (C18:0), oleic acid (C18:1) or linoleic acid (C18:2) supplied to sugar-containing media inhibit AF biosynthesis (Schultz and Luedecke, 1977). Pyiyadarshini and Tulpule reported that mixed FAs from peanuts and saturated FAs such as myristic acid (C14:0), palmitic acid (C16:0), and C18:0 promote, while unsaturated FAs such as C18:1 and C18:2 inhibit AF biosynthesis (Priyadarshini and Tulpule, 1980). Other studies showed that C18:1 and C18:2 had no effect on AF production (Fanelli et al., 1983). Thus, the role of FAs on AF biosynthesis remains controversial.

Oxylipins are known to play important roles in AF biosynthesis. In particular, it has been shown that 13*S*-hydroperoxy-9,11-octadecatrienoic acid (13*S*-HPODE) and 13*S*-hydroperoxy-9,11,15-octadecatrienoic acid (13*S*-HPOTE) inhibit, while 9*S*-HPODE, under certain conditions, promotes AF production (Burow et al., 1997). Deletion of the oxylipin-generating enzyme genes oxygenase B (*ppoB*) or *ppoC* in *A. nidulans* enhanced sterigmatocystin (ST, a precursor in AF biosynthesis pathway) production, while deletion of *ppoA* reduced ST production (Tsitsigiannis and Keller, 2006). In *A. flavus*, deletion of the lipoxygenases gene A*flox1* hampered HPODE production and AF biosynthesis *in vitro*. However, when the same strain was used to inoculate maize kernels, enhanced AF production was observed (Scarpari et al., 2014). These results imply a complex role of oxylipins in AF biosynthesis.

In this study, we re-visited the effects of saturated and polyunsaturated FAs on AF biosynthesis in *A. flavus*, and observed that both C18:0 and C18:3 promoted AF production. However, following exposure to air C18:3, but not C18:0, inhibited AF production. Detailed studies revealed that the inhibitory effect was generated in C18:3 after exposure to air for several hours. The inhibition occurred through suppressed expression of genes in the AF biosynthetic gene cluster, together with enhanced mycelial growth,

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sporulation, and kojic acid production. Mass spectrometry analyses of the autoxidated C18:3 fractions with inhibitory effects led to the identification of 15 oxylipin species.

## 2. Material and methods

### 2.1. Fungal strains and growth conditions

*A. flavus* strain A3.2890 was obtained from the China General Microbiological Culture Collection Center at the Institute of Microbiology, Chinese Academy of Sciences (Yan et al., 2012); the Papa 827 strain was provided by Prof. G.A. Payne from North Carolina State University; the NRRL 3357 strain was obtained from USDA-ARS, Peoria, Illinois. A3.2890 was used in all experiments, unless otherwise specified. To initiate cultures, spore suspensions and media were prepared as described previously (Yan et al., 2012), with an initial spore density of $10^6$ spores/mL. All cultures were grown in continuous darkness, at 28 °C, either statically when cultured on plates, or on a shaker at 180 rpm when cultured in liquid media.

### 2.2. FA assays

C18:0 (catalog number S4751) and C18:3 (62160) were purchased from Sigma–Aldrich (St. Louis), with purities above 99% and 98.5%, respectively. One hundred-fold stock solutions were used to prepare the aliquots for the experiments; 200 μL of a stock solution was transferred to a conical flask, and these flasks were then placed either in a laminar flow cabinet or in a vacuum freeze dryer (50 mbar; ModulyoD, Thermo) for 4 h, to allow the ethanol to evaporate completely. 18 mL of liquid glucose mineral salts (GMS) medium and 2 mL of spore suspension ($10^7$ spores/mL) were added to each flask and cultured. For the growth measurements, mycelia cultured in liquid GMS media with 1.25 mM air-dried C18:0, C18:3, or without exogenous FAs were harvested by filtration on days 2, 3 and 4, and then dried in a freezer–drier prior to weighing. AF content in media was analyzed with thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC), as described previously (Yan et al., 2012). To examine the effects of FAs on sporulation, 5 mg C18:0 or C18:3 was dissolved in 200 μL ethanol (final concentration 25 mg/mL), spotted on 1 $cm^2$ filter paper discs and air-dried in a laminar flow chamber for 4 h before being placed on the surface of solid two-layer GMS media, as described previously (Tsitsigiannis and Keller, 2006). After 3 days of culturing, the top-layer medium was collected using a cell scraper, and the total numbers of spores were counted under a microscope with a haemacytometer.

### 2.3. Measurements of glucose, kojic acid, and NOR content

After mycelia were cultured in liquid GMS media with 1.25 mM air-dried C18:3 or C18:0, or media lacking exogenous FA for 2, 3, or 4 days, samples were collected by passing the media first through filter paper and then through a 0.22-μm hydrophilic nylon filter. The glucose content in media was measured using a glucose determination kit (APPLYGEN, Beijing). The kojic acid content was determined according to Bentley (1957). The norsolorinic acid (NOR) content was measured using solid potato-dextrose agar (PDA) media as described previously (Hua et al., 1999).

### 2.4. Real-time PCR analyses

Total RNA was extracted from mycelia after growth in liquid GMS media with 1.25 mM air-dried C18:3 or C18:0, or media lacking exogenous FA for 3 days, using previously described methods (Zhang et al., 2014). Poly(A) mRNA was purified using a PolyATrack mRNA Isolation Kit (Promega, Madison), and cDNA was synthesized using a ReverTra Ace-α-® Kit (Toyobo, Japan). Real-time PCR was performed using SYBR Green I mix in a Rotor-Gene 3000 Cycler (Corbett Research, Australia), with primers and cycling programs listed in Table 1. Relative expression levels were quantified by comparing the expression of target genes with the expression of *β-tubulin*.

### 2.5. Oxylipin analyses

Air- and vacuum-dried C18:3 samples were analyzed using a liquid chromatography system coupled to a quadrupole time-of-flight mass spectrometer (LC/Q-TOF-MS). For the activity assays, autoxidated C18:3 was first separated with semi-preparative HPLC (Agilent 1200, Waldbronn, Germany) using a reverse phase C18 column (4.6 mm × 150 mm, 5 μm, Agilent); fractions were collected each minute from the 9th to the 15th min. These fractions were dried completely under nitrogen gas and tested in *A. flavus* assays for their effect on AF production, as described above. Individual fractions were characterized further in an ultra-fast LC system (Shimadzu, Kyoto) equipped with a C18 column (3 × 150 mm, 3.5 μm, Eclipse XDB, Agilent). The mobile phase was prepared by mixing solution A (water with 5 mM ammonium acetate and 0.02% formic acid) and solution B (water: acetonitrile, 5: 95, with 5 mM ammonium acetate and 0.02% formic acid) in a multi-step linear gradient of 20% solution B at 0–0.5 min, 20–35% solution B at 0.5–60 min; 35–50% solution B at 60–90 min, 50–70% solution B at 90–95 min; and 70–100% solution B at 95–115 min and 100% solution B at 115–120 min, with a flow rate of 0.6 mL/min. The sample injection volume was 3 μL. MS/MS analyses of autoxidated C18:3 were performed with a hybrid Q-TOF-MS equipped with an ESI source (Triple TOF™ 5600+, AB SCIEX, Foster City, CA): one TOF MS survey scan was followed by 8 MS/MS scans. The mass range for the MS scan was set to $m/z$ 100–600. For the MS/MS scans, the range was set at $m/z$ 50–600. Instrumental conditions for the MS/MS experiments were: ion spray voltage, −4500 V; ion source gas 1 (nebulizer gas), 55 psi; ion source gas 2 (heater gas), 55 psi; temperature, 550 °C; curtain gas, 30 psi;

**Table 1**
List of primers and PCR cycling programs used in this study.

| Genes | Primers used | PCR amplification schemes |
|---|---|---|
| *aflO* | F: GTCGCATATGCCCCGGTCGG<br>R: GGCAACCAGTCGGGTTCCGG | 94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles |
| *aflR* | F: AGCACCCTGTCTTCCCTAA<br>R: CTGGTCTTCCTCATCCACA | 94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles |
| *aflS* | F: CGAGTCGCTCAGGCGCTCAA<br>R: GCTCAGACTGACCGCCGCTC | 94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles |
| *β-tubulin* | F: CCAAGAACATGATGGCTGCT<br>R: CTTGAAGAGCTCCTGGATGG | 94 °C, 30 s, (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) for 40 cycles |
| *cypA* | F: CGGCGGTCCCTCTTTTCCCG<br>R: TGCGTCGTGAGGTCGGAGGT | 94 °C, 30 s, (94 °C, 30 s; 62.5 °C, 30 s; 72 °C, 30 s) for 40 cycles |
| *ordA* | F: CAACGACGCGCAGCTGGGTA<br>R: TCATGCCCCACCCCGCCATA | 94 °C, 30 s, (94 °C, 30 s; 62.5 °C, 30 s; 72 °C, 30 s) for 40 cycles |

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declustering potential, −65 V; collision energy for TOF MS, −10 V, and CE for TOF MS/MS, −35 V, with CE spread of 15 V. All samples were analyzed in a negative ionization mode. Data analyses were performed using PeakView™ software V.1.2 (AB SCIEX, Foster City, CA).

## 3. Results

### 3.1. Air-dried C18:0 promoted, while air-dried C18:3 inhibited AF production

To examine the effect of FAs on AF production, certain amounts of C18:0 and C18:3 were dissolved in ethanol to make 100-fold stock solutions and aliquoted into culture flasks. After air-drying for 4 h in a laminar flow cabinet to allow ethanol to evaporate completely, GMS media and freshly prepared spores were added and cultured as described (Yan et al., 2012), to yield final concentrations of these FAs of 0, 0.01, 0.1, 0.75, 1.25 and 5 mM. After 3-day incubations, TLC analyses were performed to examine the AF content in media. We observed that treatments with 0.1, 0.75 or 1.25 mM C18:0 promoted (Fig. 1A), while treatments with 1.25 or 5 mM C18:3 inhibited AF biosynthesis (Fig. 1B). In all subsequent experiments, the effective concentration of 1.25 mM was used. Time course analyses showed that no AF production was detected in the presence of 1.25 mM C18:3 during the 6-day culture period, while in the presence of C18:0 at the same concentration, AF production was observed on the 2nd day and the maximal AF production was observed on the 4th day (Fig. S1A).

To address whether the inhibitory effect of air-dried C18:3 on AF biosynthesis is effective in other *A. flavus* strains, the NRRL 3357 strain that only produces AFB1 and AFB2 was tested. As shown in Fig. 1C, the promoting effect of air-dried C18:0 and the inhibiting effect of air-dried C18:3 on AF production were observed consistently the various strains tested.

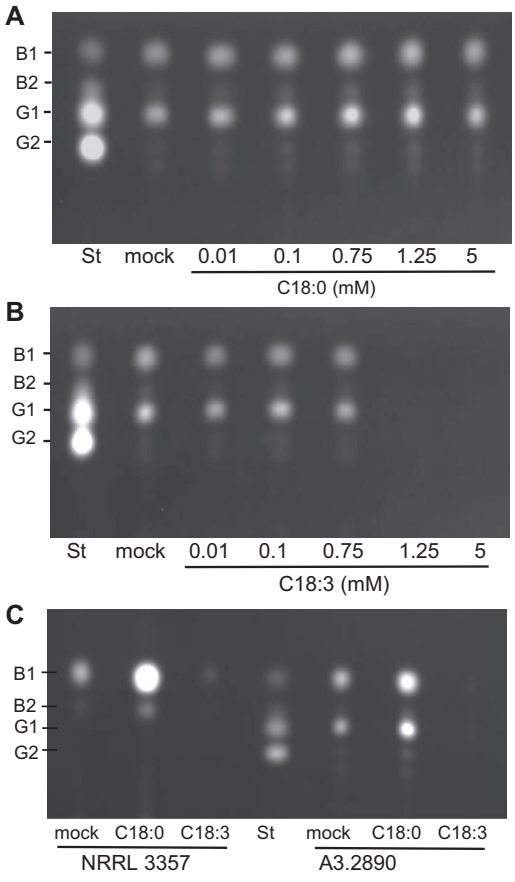


**Fig. 1.** Effects of air-dried C18:3 and air-dried C18:0 on AF production in *A. flavus*. (A) TLC plate showing AFs produced by *A. flavus* strain A3.2890 when cultured in GMS media with 0.01, 0.1, 0.75, 1.25, or 5 mM air-dried C18:0 for 3 days. Note the enhanced AF production when 0.1, 0.75, 1.25 or 5 mM C18:0 were applied. (B) TLC plate showing AFs produced by *A. flavus* strain A3.2890 when cultured in GMS media with 0.01, 0.1, 0.75, 1.25, or 5 mM air-dried C18:3 for 3 days. Note that no visible AF production was observed when 1.25 or 5 mM C18:3 were applied. (C) TLC plate showing inhibited AF production in the *A. flavus* strains A3.2890 and NRRL 3357 after growth in media with 1.25 mM air-dried C18:3 for 3 days. Enhanced AF production was observed in media with 1.25 mM air-dried C18:0. St, mixed AFB1, AFB2, AFG1 and AFG2 standards.

### 3.2. Autoxidated C18:3 enhanced mycelial growth and sporulation

Time-course experiments were performed with *A. flavus* strain A3.2890 to examine in detail the effects of air-dried C18:0 and

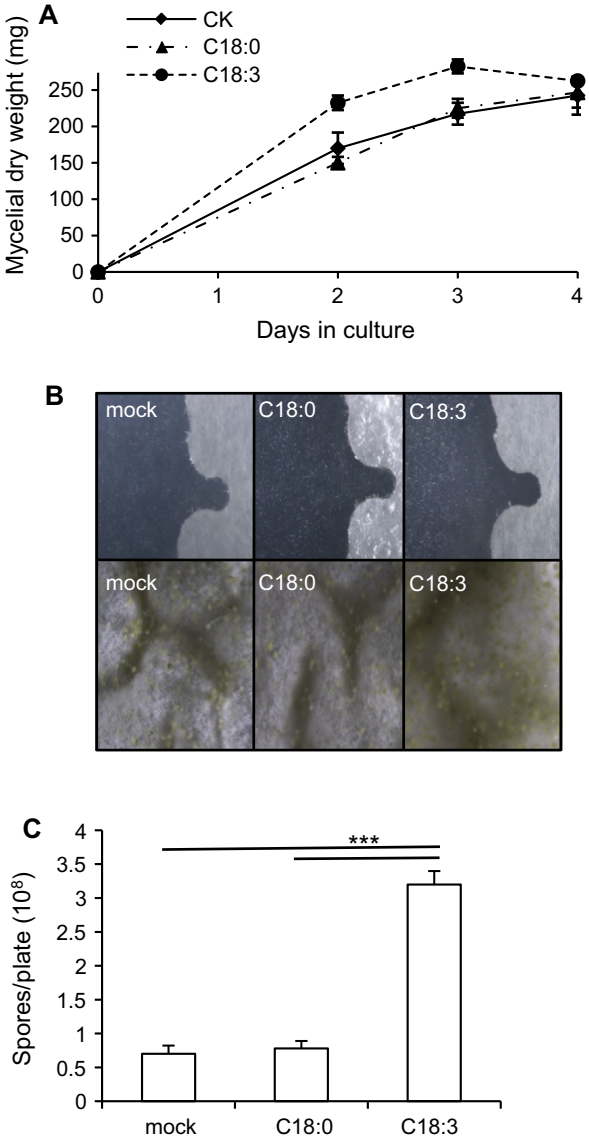


**Fig. 2.** Effects of air-dried C18:0 and C18:3 on mycelial growth and spore production in *A. flavus*. (A) Growth curves of *A. flavus* A3.2890 cultured in GMS liquid media with air-dried C18:0, C18:3, or media lacking FAs ($n = 4$ each). Note that air-dried C18:3 promoted mycelial growth. (B) Pictures showing mycelial growth and sporulation on solid GMS media with 5 mg C18:3 or C18:0 spotted on filter paper discs and air-dried. Top panel, 24 h; bottom panel, 72 h. (C) Numbers of spores produced after 72-h incubation on solid GMS media with air-dried C18:0, air-dried C18:3, or media lacking FA (mock) ($n = 4$ each). Asterisks indicate values with significant differences from the control lacking FAs, based on two-tailed Student's *t*-tests (***$P < 0.001$).

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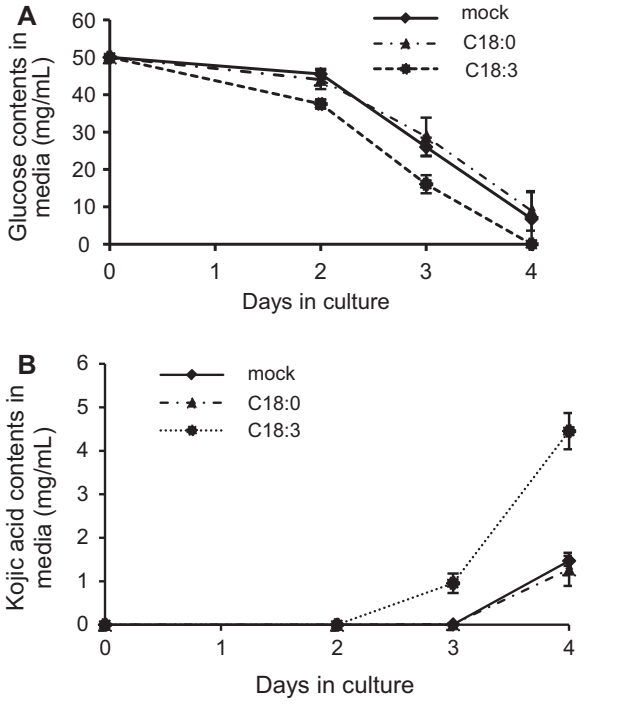


**Fig. 3.** Effects of air-dried C18:0 and C18:3 on glucose consumption and kojic acid production. (A) Glucose content in media after *A. flavus* growth in the presence of air-dried C18:0 and air-dried C18:3 for 4 days ($n = 4$ each), showing that 1.25 mM air-dried C18:3 promoted glucose consumption. (B) Kojic acid content in media after *A. flavus* growth in the presence of air-dried C18:0 and air-dried C18:3 for 4 days ($n = 4$ each), showing that 1.25 mM air-dried C18:3 enhanced kojic acid production. All values are means ± S.D.

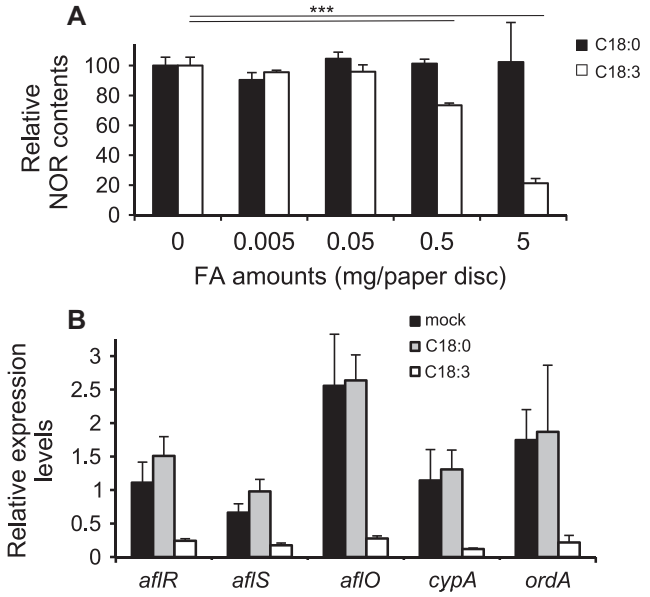


**Fig. 4.** Effects of air-dried C18:0 and air-dried C18:3 on the AF biosynthesis pathway. (A) Relative NOR contents in cultures with different concentrations of air-dried C18:0 or C18:3 spotted on filter paper discs ($n = 3$). Note that 5 mg air-dried C18:3 inhibited NOR production effectively. Asterisks indicate values with significant differences from the control lacking FAs, based on two-tailed Student's *t*-tests (***$P < 0.001$). (B) Relative expression levels of genes encoding transcriptional regulators (*aflR* and *aflS*) and AF biosynthesis enzymes (*aflO*, *cypA* and *ordA*) in the AF biosynthetic gene cluster of *A. flavus* cultured in GMS media with air-dried C18:0, C18:3, or media lacking FA (mock) ($n = 3$), as quantified by comparison to the expression level of *β-tubulin*.

C18:3 on mycelial growth. In the presence of air-dried C18:0, the mycelial growth, as measured by the mycelial dry weight, was about the same as that of the control cultures lacking exogenous FA (Fig. 2A). However, air-dried C18:3 promoted mycelial growth, as indicated by the increased mycelial dry weights in the media with air-dried C18:3 on the 2nd and the 3rd days (Fig. 2A), and a slight reduced mycelial dry weight was observed on the 4th day (Fig. 2A), suggesting early mycelial degeneration when cultured in the presence of air-dried C18:3. Apparently, the inhibition of AF biosynthesis by air-dried C18:3 does not result from inhibited fungal growth.

To assess if air-dried C18:0 or C18:3 supplied in media affected sporulation, 5 mg C18:0 and C18:3 were dissolved in 200 μL ethanol, spotted on filter paper discs and dried in a laminar flow cabinet for 4 h to allow ethanol to evaporate completely. These discs were then placed onto the top layer of solid GMS media in which spores of *A. flavus* had been mixed. Enhanced mycelial growth was observed around the filter paper supplied with air-dried C18:3 after 24 h (Fig. 2B, top panel). After 72-h of incubation, significantly more sporangia were observed on media with air-dried C18:3 as compared to media with air-dried C18:0 or media lacking FA (Fig. 2B). Spore counting indicated that the *A. flavus* grown on media with air-dried C18:3 for 3 days produced about 5-fold more spores than did samples grown on media with air-dried C18:0 or media lacking FA (Fig. 2C).

### 3.3. Autoxidated C18:3 promoted glucose consumption and kojic acid production

Since glucose was the only carbohydrate in the culture media, its consumption can be used to indicate the metabolic activity of mycelia. We measured the glucose content in media during the incubation, and observed that there was significantly less glucose from the 2nd day onwards in the culture with the air-dried C18:3 than in the controls (Fig. 3A). The glucose was completely depleted by the 4th day from the medium with air-dried C18:3, while substantial amounts of glucose were still detectable in the medium supplied with the air-dried C18:0, and in the medium without any FA (Fig. 3A).

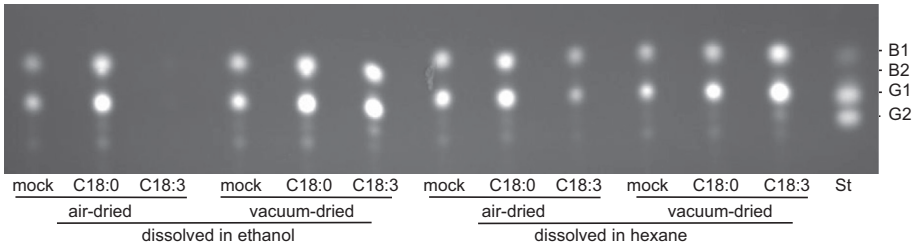


**Fig. 5.** The inhibitory effect of air-dried C18:3 on AF biosynthesis was generated during exposure to air. A TLC picture showing enhanced AF production by *A. flavus* cultured in the presence of 1.25 mM vacuum-dried C18:0 or C18:3 for 3 days. However, when dried in air, C18:0 still promoted, while C18:3 inhibited AF production, no matter whether ethanol or hexane was used as the solvent.

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To assess if air-dried C18:3 repressed other secondary metabolic pathways, we examined kojic acid production during 4-day cultures and found that the kojic acid content in the culture media supplied with the air-dried C18:3 was increased (Fig. 3B). No significant difference in koic acid content was observed between media with air-dried C18:0 and media lacking FA (Fig. 3B).

### 3.4. Autoxidated C18:3 inhibited the AF biosynthesis pathway through suppression of the expression of genes in the AF biosynthetic gene cluster

To examine if the AF biosynthetic pathway is inhibited by the air-dried C18:3, experiments were performed using the mutant *A. flavus* strain Papa 827 in which AF biosynthesis pathway is blocked after the production of the first stable intermediate, NOR. We observed that, as compared to samples grown on media with air-dried C18:0 or lacking FA, mycelia grown on media with air-dried C18:3 showed greatly reduced NOR accumulation (Fig. 4A), suggesting that autoxidated C18:3 blocks the entire AF biosynthetic pathway.

Real-time PCR analyses were then performed with *A. flavus* strain A3.2890 cultures to examine the expression of the genes encoding transcriptional regulators (*aflR* and *aflS*) and AF biosynthetic enzymes (*afl0*, *cypA* and *ordA*) in the AF biosynthetic gene cluster (Yu et al., 2011). The expression of all of these genes was suppressed in the cultures with air-dried C18:3 (Fig. 4B). No significant difference in the expression of any of these genes was observed in cultures grown in media with air-dried C18:0 or media lacking FA (Fig. 4B). These results suggest that autoxidated C18:3 inhibits the AF biosynthesis pathway at the transcriptional level.

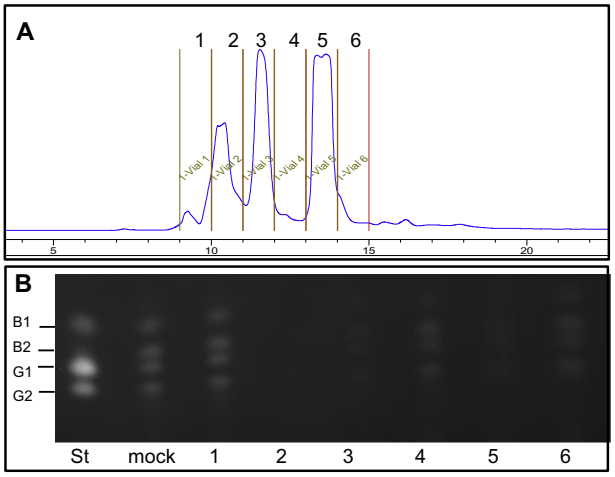


**Fig. 6.** Fractionation and activity assays of fractionated air-dried C18:3. (A) An HPLC chromatograph of 6 fractions collected from the 9th to the 15th min after air-dried C18:3 was separated by semi-preparative HPLC. (B) TLC plate showing that AF production in *A. flavus* strain A3.2890 was strongly inhibited by fractions 2, 3, and 5. AFs were extracted from media after 3-day cultures. St, mixed AFB1, AFB2, AFG1 and AFG2 standards.

### 3.5. The inhibitory effect of C18:3 on AF production was generated during exposure to air

We then addressed if the inhibitory effect of C18:3 on AF biosynthesis is caused by exposure to air, and observed that when the ethanol-dissolved C18:3 was dried under vacuum the

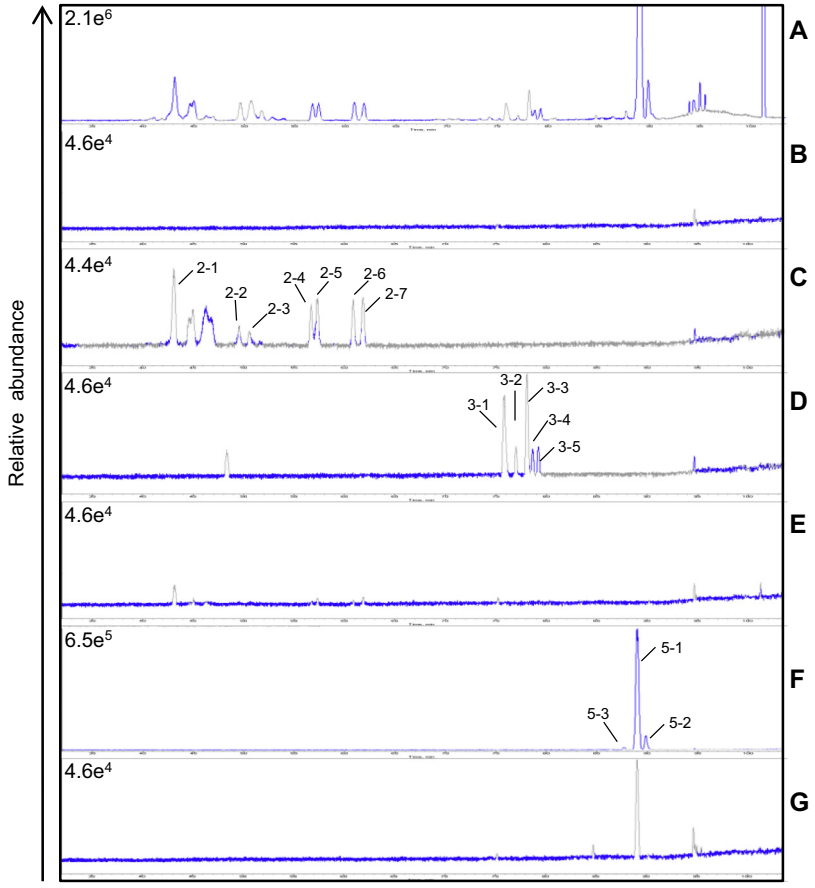


**Fig. 7.** LC/Q-TOF-MS analyses of air-dried C18:3 fractions. (A) A TIC plot showing compound profiles of the air-dried C18:3. (B–G) TIC plots of fractions 1–6 obtained after the semi-preparative HPLC separation, respectively, showing profiles of each fraction. Numbers next to the peaks mark compounds identified in different fractions.

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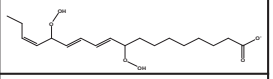
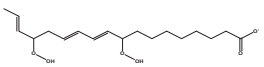
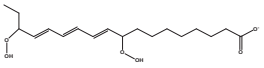
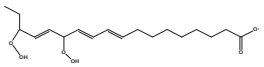
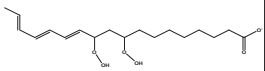
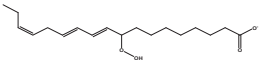
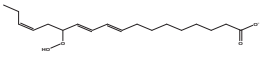
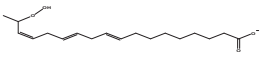
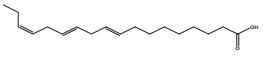
**Table 2**
Putative oxylipins identified from autoxidated C18:3.

| Peaks | Candidate oxylipins | Candidate structure | Formula and molecular mass | Retention time (min) | Relative content to total oxylipins |
|---|---|---|---|---|---|
| 2-1 | 9,14-diHPOTE |  | $C_{18}H_{30}O_6$ (342.2042) | 43.1 | 10.1% |
| 2-2 | 9,15-diHPOTE |  | | 49.6 | 4.0% |
| 2-3 | | | | 50.7 | 6.6% |
| 2-4 | 9,16-diHPOTE |  | | 56.7 | 4.1% |
| 2-5 | | | | 57.4 | 4.0% |
| 2-6 | | | | 60.9 | 4.5% |
| 2-7 | | | | 61.8 | 4.2% |
| 3-1 | 13,16-diHPOTE |  | | 75.9 | 3.7% |
| 3-3 | | | | 78.1 | 5.0% |
| 3-4 | | | | 78.7 | 2.6% |
| 3-2 | 9,11-diHPOTE |  | | 77 | 0.8% |
| 3-5 | | | | 79.3 | 1.9% |
| 5-1 | 9-HPOTE |  | $C_{18}H_{30}O_4$ (310.2150) | 89.0 | 43.8% |
| 5-2 | 13-HPOTE |  | | 89.9 | 2.3% |
| 5-3 | 17-HPOTE |  | | 87.75 | 2.4% |
| parent | Linolenic acid (C18:3) |  | $C_{18}H_{30}O_2$ (278.2246) | 101.3 | N/A |

inhibitory effect disappeared. Instead, intriguingly, a promoting effect was observed (Fig. 5). Similar results were obtained when C18:3 was dissolved in hexane (Fig. 5). The promoting effect of C18:0 on AF production was observed consistently when hexane was used as the solvent, no matter whether the FA was dried in air or dried under vacuum (Fig. 5). Studies using different air exposure times showed that the inhibitory effect in AF production was evident when C18:3 was exposed for longer than 2 h; complete inhibition of AF production was observed in cultures treated with C18:3 that had been exposed to air for 16 h or longer (Fig. S1B). These observations together suggest that the inhibitory effect of C18:3 on AF production is generated during exposure to air.

### *3.6. Air-dried C18:3 inhibited AF production through oxylipin species produced by autoxidation*

To elucidate what happened to C18:3 during the exposure to air, we profiled air-dried and vacuum-dried C18:3 samples with LC/Q-TOF-MS, and found several peaks in the air-dried C18:3 sample, which were not present in the vacuum-dried C18:3 sample (Fig. S2A–C, indicated by blue bars). We then fractionated the air-dried C18:3 sample using semi-preparative HPLC (Fig. 6A). Activity assays were performed for all 6 fractions obtained. Fractions 2, 3, and 5 effectively inhibited AF biosynthesis; fractions 4 and 6 showed weak inhibition; no inhibitory effect was observed for fraction 1 (Fig. 6B).

To characterize the chemical identities of compounds generated by autoxidation, the unfractionated air-dried sample and all 6 fractionated samples were analyzed with LC/Q-TOF-MS and compared (Fig. 7B–G) (Fig. 6A). We observed that, as can be seen in the total ion chromatogram (TIC) plots, no evident peak was detected in fraction 1 (Fig. 7B). Fractions 2, 3, and 5 showed multiple compound peaks (Fig. 7C, D and F), while one weak peak overlapping with peak number 2–1 of fraction 2 was detected in fraction 4 (Fig. 7E) and one peak overlapping with peak number 5–1 in fraction 5 was detected in fraction 6 (Fig. 7G). The relative ratios of these individual compounds are shown in the last column of Table 2.

Accurate MS analyses of all peaks (2–1, 2–2, 2–3, 2–4, 2–5, 2–6 and 2–7) detected in fraction 2, together with their isotope

abundance ratios, revealed that these seven compounds are isobaric with an *m/z* value of 341.197. Using the Formula Finder tool in Peak View™ software, the putative molecular formula of these molecules was identified as $C_{18}H_{30}O_6$ (with the theoretical *m/z* value $[M–H]^-$: 341.1970, and observed *m/z* values: 341.1972 (2–1), 341.1972 (2–2), 341.1968 (2–3), 341.1965 (2–4), 341.1978 (2–5), 341.1975 (2–6) and 341.1968 (2–7) (Fig. 8; Figs. S3A, S4 and S5). Similarly, five compounds (peaks 3–1, 3–2, 3–3, 3–4 and 3–5) in fraction 3 were identified to have the same molecular formula as those in fractions 2, $C_{18}H_{30}O_6$ (with the observed *m/z* values: 341.1969 (3–1), 341.1964 (3–2), 341.1961 (3–3), 341.1979 (3–4), 341.1974 (3–5) (Figs. S3B, S6 and S7). In contrast, three compounds (peaks 5–1, 5–2 and 5–3) in fraction 5 were found to have a molecular formula of $C_{18}H_{30}O_4$ (with the theoretical *m/z* value $[M–H]^-$: 309.2071, and observed *m/z* values: 309.2064 (5–1), 309.2068 (5–2), 309.2073 (5–3) (Figs. S3C, S8–S10). One major compound peak was identified in fraction 6 (Fig. 7G); it had the same retention time and the same molecular formula as peak 5–1 in faction 5, but with much lower abundance (about 1/20) (Fig. 7F).

MS/MS spectra of these compounds were obtained (Fig. 8, Figs. S4–S10), and analyzed using the Fragment Pane tool in combination with spectral information for standard compounds available in public databases. In principle, C18:3 [$CH_3CH_2CH$=C HCH$_2$CH=CHCH$_2$CH=CH(CH$_2$)$_7$COOH] with two pentadienyl structures may produce two radicals after the abstraction of the hydrogen atoms at the C—11 and C—14 positions. The addition of oxygen may occur at either end of these two pentadienyl structures, or at these two radicals (C—11 or C—14), producing different

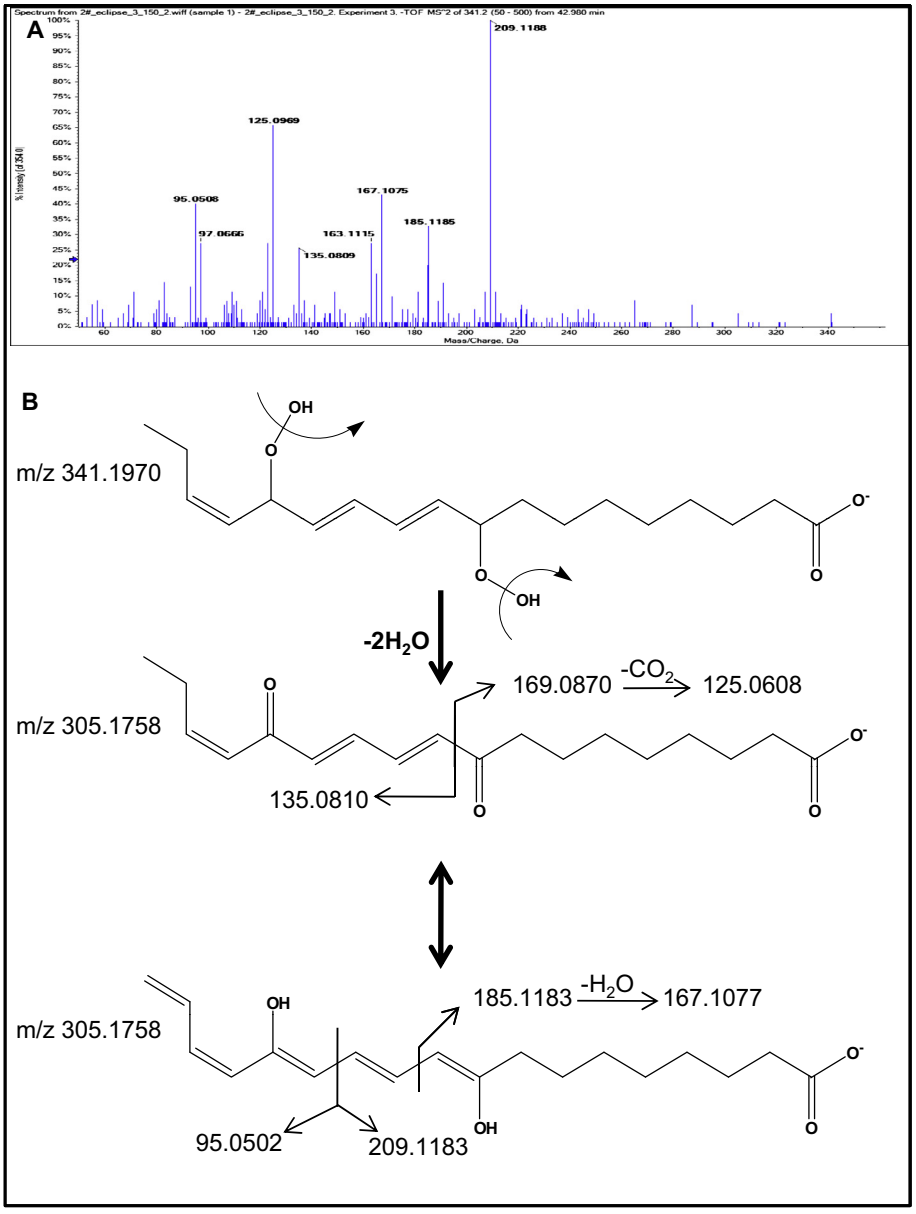



**Fig. 8.** MS/MS characterization of the compound peak 2–1 in fraction 2 produced in C18:3 after autoxidation. (A) MS/MS spectrum of peak 2–1, showing fragments produced from the parent ion *m/z* 341.1972. Note fragment ions generated were *m/z* 209.1188, *m/z* 185.1185, *m/z* 167.1075, *m/z* 135.0809, *m/z* 125.0969, and *m/z* 95.0508. (B) The putative identity of peak 2–1 is 9,14-diHPOTE, based on expected fragmentation pattern and keto-enol tautomerism of 9,14-diHPOTE (theoretical *m/z* 341.1790) in the MS/MS analysis. Note that the fragment ion *m/z* 305.1758 was produced by dehydration of two peroxy bonds of the parent ion; fragment ions *m/z* 209.1183, *m/z* 185.1183, *m/z* 135.0810, and *m/z* 95.0502 were formed by heterolytic or homolytic cleavage of the C9—C10, C10—C11, and C12—C13 (outside the double bonds) in the fragment ion *m/z* 305.1758; *m/z* 125.0608 was formed by elimination of $CO_2$ from fragment ion *m/z* 169.0870; and *m/z* 167.1077 was formed by elimination of $H_2O$ from fragment ion *m/z* 185.1183.

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forms of hydroperoxy-C18:3 (Porter et al., 1995). Accordingly, the MS/MS spectrum of the peak 2–1 (*m*/*z* 341.1962) showed fragment ions of *m*/*z* 209.1188, 185.1185, 167.1075 (185.1183 – $H_2O$), 135.0809, 125.0969 (169.0870 – $CO_2$) and 95.0508 (Fig. 8A), and thus the compound was putatively identified as 9,14-diHPOTE. A proposed fragmentation pattern of the compound is shown in Fig. 8B. In a similar manner, all peaks marked in the TIC plots of fractions 2 and 3 were identified as different structural isomers of diHPOTE, with two hydroperoxy moieties attached to different carbon atoms (Figs. S4–S7). Some of these peaks, such as 2–4 to 2–7, were stereo isomers of one structure, with almost the same MS and MS/MS fragmentation patterns (Table 2). Three compounds in fraction 5 were identified as structural isomers of monoHPOTE, with one hydroperoxy moiety attached to either the 9th, 13th, or 17th carbon (Table 2), based on their fragmentation patterns (Figs. S8–S10). In summary, 15 compounds with 8 different structures were identified in the air-dried C18:3 as stereo or structural isomers of mono- and di-HPOTE, although our present analyses did not allow us to determine the *R* or *S* configurations of the hydroperoxy groups.

## 4. Discussion

In this study, the effects of various FAs on AF production were examined in *A. flavus*. We showed that both the saturated fatty acid C18:0 and the polyunsaturated fatty acid C18:3, if dried under vacuum, promoted AF biosynthesis. However, after exposure to air for 4 h or longer, C18:3 inhibited, while C18:0 still promoted AF production. Expression analyses revealed that autoxidated C18:3 inhibited AF biosynthesis through suppressed expression of genes in the AF biosynthetic gene cluster. Fractionation and MS/MS analyses suggested that the inhibition occurs through oxylipin species produced during autoxidation.

It is well-known that seeds with high FA content are more susceptible to AF accumulation when infected by AF-producing *Aspergillus* strains (Fabbri et al., 1980; Reddy et al., 1992). FA-rich aleurone and embryos in maize seeds accumulate more AFs than starchy endosperms following infection by *Aspergillus* (Brown et al., 1993; Keller et al., 1994), which suggests that FAs promote AF biosynthesis. However, *in vitro* studies with FAs have yielded controversial results with respect to AF production. Firstly, FAs cannot be utilized effectively by *Aspergillus* as a sole carbohydrate source (Davis and Diener, 1968; Fanelli and Fabbri, 1980; Gao and Kolomiets, 2009). Secondly, when FAs are supplied to sugar-containing media, both promoting and inhibiting effects on AF biosynthesis have been observed in different reports (Schultz and Luedecke, 1977; Priyadarshini and Tulpule, 1980; Tiwari et al., 1986). The findings of this study showed that both C18:0 and C18:3 promoted AF biosynthesis. However, after air exposure for several hours, C18:3 inhibited AF production, while C18:0 still promoted AF production. Expression analyses showed that the inhibition acts through the suppressed expression of the genes encoding transcriptional regulators and AF biosynthesis enzymes in the AF biosynthetic gene cluster. The striking difference between autoxidated and non-autoxidated C18:3 on AF biosynthesis may explain why controversial results were observed in the past (Gupta et al., 1974; Fanelli and Fabbri, 1980; Tiwari et al., 1986), as was proposed previously (Fanelli and Fabbri, 1980). Methyl jasmonate, as a lipid oxidation product, has been shown to inhibit AF biosynthesis (Goodrich-Tarikulu et al., 1995). In principle, if C18:3 has been intentionally or unintentionally exposed to air for several hours during storage or handling, autoxidation may occur and one would expect to see a resulting inhibition of AF biosynthesis in *Aspergillus* cultures. The inhibitory effect acts neither through reactive oxygen species (ROS), nor through hydroxylated or hydroperoxy FAs, since both types of compounds are known to promote AF biosynthesis (Fanelli et al., 1983; Jayashree and Subramanyam, 2000; Reverberi et al., 2006). It is known that kojic acid is a potent antioxidant that is able to scavenge ROS (Gomes et al., 2001). In this study, we observed a negative correlation between kojic acid production and AF biosynthesis when air-dried C18:3 was applied, which is similar to previous reports with several other treatments (Jayashree and Subramanyam, 1999; Kim et al., 2008; Tzanidi et al., 2012; Zhang et al., 2014). Therefore, we believe that autoxidated C18:3 may function as a signal molecule to repress the AF biosynthetic pathway at the transcriptional level.

Further, we observed that autoxidated C18:3 promoted sporulation in *A. flavus*, which is in agreement with reports showing that exogenously supplied hydroxy-C18:2, 9S-HPODE and 13S-HPODE enhance asexual spore formation (Calvo et al., 1999; Tsitsigiannis et al., 2005b; Brown et al., 2009). When genes encoding the oxylipin-generating enzymes *ppoA*, *ppoB*, *ppoC*, *ppoD*, and *lox* are downregulated in *A. flavus*, the production of asexual spores decreased, while the formation of sclerotia (hardened and pigmented mycelial tissue, with the capacity of long dormancy during unfavorable conditions) increased (Brown et al., 2009). In *A. nidulans*, it has been shown that endogenous C18:1- and C18:2-derived psi factors (fungal oxylipins) regulate the ratio of asexual to sexual spores (Tsitsigiannis et al., 2005a).

In this study, fractionations, activity assays and mass spectrometry analyses with autoxidated C18:3 led us to identify 15 putative compounds with 8 different structures including 9-HPOTE, 13-HPOTE, 17-HPOTE, 9,11-diHPOTE, 9,14-diHPOTE, 9,15-diHPOTE, 9,16-diHPOTE, and 13,16-diHPOTE. Although oxylipin species produced from the autoxidation of polyunsaturated FAs have been reported previously (Gardner, 1991), the exact structures of oxylipins produced from C18:3 autoxidation have not been characterized before. *In vitro* studies in *A. parasiticus* have shown that 13S-HPODE and 13S-HPOTE inhibits AF production, while 9S-HPODE enhances AF production at certain time points by extending the expression time of AF biosynthetic genes (Burow et al., 1997). Enhanced AF production has also been reported when *A. parasiticus* is grown in the presence of hydroxyl-C18:1 (Fabbri et al., 1983; Passi et al., 1984) as well as with a combination of C18:2 and soybean LOX-1 (Hamberg and Samuelsson, 1967). We did not observe any promoting effect from any of these autoxidated C18:3 fractions in this study. The activities of individual oxylipins need to be characterized in the future. We cannot exclude the possibility that different oxylipin species act on the AF biosynthetic pathway differently.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.11.005.

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