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ORIGINAL ARTICLE

Linolenate 9*R*-Dioxygenase and Allene Oxide Synthase Activities of *Lasiodiplodia theobromae*

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Abstract Jasmonic acid (JA) is synthesized from linolenic acid (18:3n-3) by sequential action of 13-lipoxygenase, allene oxide synthase (AOS), and allene oxide cyclase. The fungus *Lasiodiplodia theobromae* can produce large amounts of JA and was recently reported to form the JA precursor 12-oxophytodienoic acid. The objective of our study was to characterize the fatty acid dioxygenase activities of this fungus. Two strains of *L. theobromae* with low JA secretion (~0.2 mg/L medium) oxygenated 18:3n-3 to 5,8-dihydroxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid as well as 9*R*-hydroperoxy-10*E*,12*Z*,15*Z*-octadecatrienoic acid, which was metabolized by an AOS activity into 9-hydroxy-10-oxo-12*Z*,15*Z*-octadecadienoic acid. Analogous conversions were observed with linoleic acid (18:2n-6). Studies using [11*S*-^{2}H]18:2n-6 revealed that the putative 9*R*-dioxygenase catalyzed stereospecific removal of the 11*R* hydrogen followed by suprafacial attack of dioxygen at C-9. Mycelia from these strains of *L. theobromae* contained 18:2n-6 as the major polyunsaturated acid but lacked 18:3n-3. A third strain with a high secretion of JA (~200 mg/L) contained 18:3n-3 as a major fatty acid and produced 5,8-dihydroxy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid from added 18:3n-3. This strain also lacked the JA biosynthetic enzymes present in higher plants.

Keywords *Botryodiplodia theobromae* · Cytochrome P450 · 9*R*-HPODE · Heme peroxidase · Jasmonic acid · Oxygenation mechanism

Abbreviations

AOS	Allene oxide synthase
CP	Chiral phase
P450	Cytochrome P450
DiHODE	Dihydroxyoctadecadienoic acid
DOX	Dioxygenase
HHDTrE	Hydroxyhexadecatrienoic acid
HPHDTrE	Hydroperoxyhexadecatrienoic acid
HOME	Hydroxyoctadecenoic acid
HPODE	Hydroperoxyoctadecadienoic acid
HPOTrE	Hydroperoxyoctadecatrienoic acid
JA	(−)-Jasmonic acid
JAs	Jasmonates
(+)-7-iso-JA	3*R*,7*S*-JA
LDS	Linoleate diol synthase
LOX	Lipoxygenase
MO	Methyloxime
NP	Normal phase
OPDA	Oxophytodienoic acid
RP	Reversed phase

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Introduction

Jasmonic acid (JA), an important signal molecule in plant defense and development, is present throughout the plant kingdom as the free acid, the methyl ester, the hydroxyl

derivatives, or amino acid conjugates [1–3]. The tropical and subtropical plant pathogen *Lasiodiplodia theobromae* (synonym *Botryodiplodia theobromae*) secretes JA [4, 5] and some strains could even be used for commercial JA production [5, 6].

JA is formed in plants sequentially from α-linolenic acid (18:3n-3) or hexadecatrienoic acid (16:3n-3) by 13*S*-lipoxygenase (13*S*-LOX), allene oxide synthase (AOS), allene oxide cyclase, 12-oxophytodienoate reductase, and three steps of β-oxidation [1–3, 7]. Key intermediates are 12-oxophytodienoic acid (12-OPDA) and dinor-12-OPDA from 18:3n-3 to 16:3n-3, respectively. JAs have a wide array of biological activities in plants (plant defense, stress adaptation and development) and can inhibit aflatoxin production and delay spore germination of *Aspergillus flavus* [8]. A recent report also suggests that JA could be of medical value as a growth suppressor of human cancer cells [9].

A biosynthetic pathway from 18:3 to 16:3 to JAs, essentially as described in plants, seems likely in fungi [6, 10], and plants and fungi form JAs with identical absolute configuration [5, 11]. JAs and the methyl ester of a key intermediate, 12-OPDA, were identified as a metabolite of 18:3n-3 in the culture medium of *L. theobromae* [10]. However, whether JA and 12-OPDA are formed by the same enzymes as in plants is unknown. LOXs are ubiquitous in plants, but only few LOXs of fungi have been identified and characterized. Unfortunately, the genome of *L. theobromae* has not yet been sequenced. Hydroperoxy fatty acids can be formed in fungi also by heme containing dioxygenases, e.g., linoleate diol synthases (LDS) and other oxygenases of the dioxygenase-cytochrome P450 (DOX-CYP) family of fusion proteins [12–16]. For comparison, *Aspergillus terreus* was recently found to express linoleic acid 9*R*-dioxygenase (9*R*-DOX) and AOS activities [17]. This 9*R*-DOX activity could be due to a heme containing dioxygenase, as LOX genes have not been identified in the genome of *A. terreus* [17].

The aim of the present study was to investigate the oxidation and further transformation of polyunsaturated fatty acids by *L. theobromae*. This fungus is a devastating plant pathogen, and its access from many fungal collection centers is restricted for environmental safety reasons. The few strains, which produce JA in large amounts [18], are also guarded by commercial interests. In this study, we used two strains of *L. theobromae* from the CBS Fungal Biodiversity Center, and a non-commercial strain with prominent JA biosynthetic capacity [19].

Materials and Methods

Materials

16:3n-3 (99%), oleic acid (18:1n-9; 99%), 12*Z*-octadecenoic acid (18:1n-6; 99%), linoleic acid (18:2n-6; 99%), U[^{13}C]18:2n-6 (98%), 18:3n-3 (99%), γ-linolenic acid (18:3n-6; 99%), and 9*Z*,12*Z*-eicosadienoic acid (20:2n-6; 99%) were from Lipidox, Sigma and Larodan, and stored as stock solutions (50–100 mM) in ethanol at −20 °C. HPLC solvents were from VWR. [11*S*-^{2}H]18:2n-6 (>95% ^{2}H) was prepared as described [20, 21]. 9-Hydroxy-10-oxo-10, 12*Z*-octadecadienoic acid was from Lipidox (Stockholm, Sweden). 11*S*- and 11*R*-hydroperoxy-7*Z*,9*E*,13*Z*-hexadecatrienoic acids (11-HPHDTrE), 9*S*-hydroperoxy-10*E*, 12*Z*-octadecadienoic acid (9*S*-HPODE), 13*R*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*R*-HPODE), 13*R*-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13*R*-HPOTrE), and 13*S*-HPODE were prepared with LOXs (tomato fruit [22], recombinant manganese LOX (Mn-LOX) [23], and soybean LOX-1 (Lipoxidase, Sigma)). 11*S*- and 11*R*-HOME(12*Z*) were prepared with PGH synthase-1 and Mn-LOX [24, 25]. A racemic mixture of HPODE was obtained by photo oxidation with methylene blue [26]. The *O*-methyloxime (MO) derivative of [5,5,7-$^{2}H_3$](−)-JA used as an internal standard during quantification of JA was synthesized by treatment of (−)-JA with Na_2CO_3 in D_2O followed by preparation and isolation of the MO derivative. Solvents for HPLC and other chemicals were of analytical grade and obtained from VWR and Sigma-Aldrich. *L. theobromae* (CBS 117454; CBS 122127) were from CBS Fungal Biodiversity Center (Baarn, Delft, The Netherlands). The *B. theobromae* strain 2334 was isolated in Cuba, characterized and grown as described [19], and it will be referred to as *L. theobromae* strain 2334.

Fungal Growth

The two CBS strains were grown on agar slants (15 g agar/2 g malt extract/0.75 g $NaNO_3$/0.35 g $MgSO_4{\cdot}7H_2O$/5 g sucrose) at 27 °C for 3–4 days and then stored at +4 °C. For analysis, a piece of the agar slants of *L. theobromae* were grown in a modified Czapek-Dox medium (per liter: 2 g KH_2PO_4, 0.6 g $MgSO_4{\cdot}7H_2O$, 0.3 g KCl, 7.5 g $NaNO_3$, 50 g sucrose, 1 ml trace metals ([27]); pH was adjusted to 5.5 with KOH) at 27 °C without shaking for 10–12 days. The *L. theobromae* strain 2334 was grown in malt extract agar slant at 30 °C for 3 days and then stored at 4 °C; for analysis, a piece of the agar slants of this fungus was grown in a medium previously described [19]. The mycelia of the strains were harvested by filtration. The filtrate was assayed for JAs, whereas the mycelia were washed with saline, blotted dry, weighed, grinded in liquid nitrogen and stored at −80 °C until analysis.

Assay of Enzyme Activity

The nitrogen powder was homogenized (glass-Teflon, 10 passes; +4 °C) in 10–20 vols. (w/v) of 0.1 mM KH_2PO_4

buffer (pH 7.4)/2 mM EDTA/0.04% Tween-20, and centrifuged at 15,000×*g* (10 min, +4 °C). The supernatant was used immediately for studies of enzyme activities. Typically, an aliquot (0.25–0.5 ml) was incubated with 80–100 μM of fatty acids or fatty acid hydroperoxides for 30–40 min on ice. The incubation was terminated with ethanol (4 vols.) and centrifuged. The supernatant was diluted with water and extracted (SepPak/C_{18}; Waters), as described [21, 28]. Triphenylphosphine was used to reduce hydroperoxy fatty acids to alcohols. The subcellular distributions of enzyme activities were determined by differential centrifugations (15,000×*g*, 10 min; +4 °C; 100,000×*g*; +4 °C; 60 min) and assay of enzyme activities in the high speed supernatant and the microsomal fractions.

LC–MS/MS

Reversed phase-HPLC (RP-HPLC) with MS/MS analysis was performed with a Surveyor MS pump (ThermoFisher), a manual or an automatic injector (Surveyor; ThermoFisher), and with an octadecyl silica column (5 μm; 150 × 2.1 mm; Phenomenex), which was eluted at 0.3 ml/min with methanol/water/acetic acid, 750/250/0.05 or 800/200/0.05. The effluent was subjected to electrospray ionization (ESI) in a linear ion trap mass spectrometer (LTQ, ThermoFisher) with analysis of negative ions. In some experiments, we also analyzed the effluent by UV absorption (photodiode array detector, path length 5 cm; Surveyor PDA plus, ThermoFisher). The heated transfer capillary was set at 315 °C and the ion isolation width usually at 1.5 (and at 5 in the first selection for MS^3 analysis of hydroperoxides [26]). The collision energy was set at 35 (arbitrary scale) and the tube lens at 90–120 V. For analysis of products formed from [$^2H_1$]18:2n-6, we used an isolation width of 6 for the carboxylate anion.

Normal phase HPLC (NP-HPLC) and chiral phase-HPLC (CP-HPLC) were performed as described in [17, 28]. Steric analysis of 10-HODE was performed with a Reprosil Chiral-NR column (8 μm; 250 × 2 mm; eluted with isopropanol/hexane/acetic acid, 3/97/0.01). The enantiomers of 9- and 13-HODE, 9-HOTRE, HOME, HHDTrE and α-ketols were resolved with Reprosil Chiral AM (5 μm; 250 × 2 mm; eluted with methanol/hexane/acetic acid or ethanol/hexane/acetic acid, 5/95/0.01, at 0.15–0.2 ml/min) [28]. The effluent was mixed with isopropanol/water, 60/40, from a second pump (Surveyor MS) [28] and subjected to ESI with MS/MS analysis of carboxylate anions.

Quantitative Determination of (−)-JA and (+)-7-iso-JA and Fatty Acid Analysis

The medium (0.05–2 mL) of the growing cultures of *L. theobromae* was diluted with water to make a total volume of 2 mL and treated with 8 mL of a methanolic solution of 30 mM methoxyamine hydrochloride. The mixture was vortexed and kept at room temperature for 15 h. The *O*-methyloxime (MO) derivative of [5,5,7-$^2H_3$](−)-JA (3 μg) was added as an internal standard, and the material obtained following extraction with diethyl ether was dissolved in chloroform/2-propanol (2:1, v/v) and applied to a Supelclean LC-NH_2 solid phase extraction cartridge (Supelco, Bellefonte, PA). Elution with diethyl ether/acetic acid (98:2, v/v) afforded the JA-MO derivative, which was methyl-esterified by treatment with diazomethane and subjected to GC–MS analysis. For this purpose, a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas-chromatograph was used. The instrument was operated in the selected ion monitoring mode using the ions *m/z* 253 (unlabeled JA-MO methyl ester) and 256 (deuterium-labeled JA-MO methyl ester). Amounts of (−)-JA and (+)-7-*iso*-JA were calculated from areas of the *m/z* 253 peaks due to these compounds and from the area of the *m/z* 256 peak due to the deuterated standard.

The fatty acid composition of *L. theobromae* was determined by GC–MS analysis after hydrolysis as described [29]. The fatty acid methyl esters were separated on a Supelcowax-10 Capillary GC Column (30 m, film thickness 0.25 μm, carrier gas helium), and the column temperature was raised from 150 °C at 5 °C/min.

Results

Fatty Acid Composition

We analyzed the fatty acid composition of *L. theobromae* strains CBS 122127 and 2334.

The four main fatty acids in the mycelium of CBS 122127 were 16:0 (37%), 18:2 (25%), 18:1 (24%), and 18:0 (14%). Traces of 20:0 were detected, but not the two expected precursor fatty acids of JA biosynthesis, 18:3n-3 and 16:3n-3. In contrast, the mycelium of *L. theobromae* strain 2334 contained 18:3n-3 as a major fatty acid, as illustrated in Fig. 1 along with the major fatty acids also found in CBS122127. 16:3n-3 was not detected.

Jasmonic Acid Production

Both commercial strains produced JAs, but (−)-JA and (+)-JA could only be detected in the growth medium in low concentrations [<0.2 μg/mL; e.g., 57 ng/mL (−)-JA, 109 ng/mL (+)-7-*iso*-JA (3*R*,7*S*-JA)]. In contrast, *L. theobromae* strain 2334 formed JAs in large amounts, about 200 μg/mL (e.g., 196 μg/mL (−)-JA and 13 μg/mL (+)-7-*iso*-JA) in agreement with previous reports [18]. In

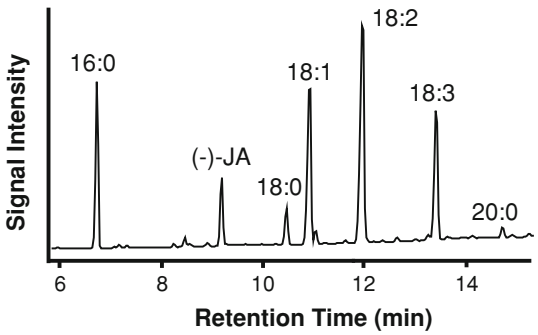

Fig. 1 GC–MS analysis of the fatty acid composition *of L. theobromae* strain 2334 with prominent JA biosynthesis. The fatty acids were analyzed as methyl ester derivatives. The commercial strain CBS 122127 lacked 18:3n-3, contained the other fatty acids of strain 2334, and formed only relatively small amounts of JA (0.2 vs 200 mg/L)

another experiment, media collected after growth of strain 2234 for 10 and 12 days were found to contain 216 μg/mL and 178 μg/mL, respectively, of (−)-JA plus (+)-7-*iso*-JA.

Oxylipin Biosynthesis by Subcellular Fractions

Subcellular fractions (10,000×*g*) of all three strains contained 5,8-LDS activity as judged from 8-hydro(per)oxy metabolites and 5,8-diols formed from 18:2n-6, [U-^{13}C] 18:2n-6, and 18:3n-3. Unexpectedly, subcellular fractions of the strain 2334 with prominent secretion of JA did not produce significant amounts of other oxylipins than 8-hydro(per)oxy metabolites and 5,8-diols, whereas the two commercial strains appeared to oxygenate fatty acids to these and to additional metabolites.

18:3n-3 and *16:3n-3* 18:3n-3 was oxidized by nitrogen powder of the two *L. theobromae* CBS strains to 5,8-DiHOTrE and to an α-ketol, 9-hydroxy-10-keto-12*Z*,15*Z*-octadecadienoic acid, as shown in Fig. 2a. The ESI–MS/MS spectrum of the α-ketol (*m*/*z* 309 → full scan) is shown in Fig. 2b. A characteristic ion with mass of even number was noted at *m*/*z* 200, likely a radical ion, which is also present in the corresponding spectrum of 9-hydroxy-10-oxo-12*Z*-octadecenoic acid [17]. Significant amounts of the corresponding γ-ketol could neither be detected by RP-HPLC nor NP-HPLC. The α-ketol was likely formed from an allene oxide, 9,10-epoxy-10,12*Z*,15*Z*-octadecatrienoic acid. Hydrolysis of this allene oxide was expected to mainly form the *S* stereoisomer, and this was confirmed (insert in Fig. 2a).

The MS/MS spectrum of 5,8-DiHOTrE was as reported [30]. The less polar metabolites were identified as a mixture of 8-H(P)OTrE, 10-H(P)OTrE, and 9-H(P)OTrE (mainly 9*R*). Although 13-HPOTrE was not detected, we investigated whether 13*S*- or 13*R*-HpOTrE were substrates of the AOS activity, but this was not the case.

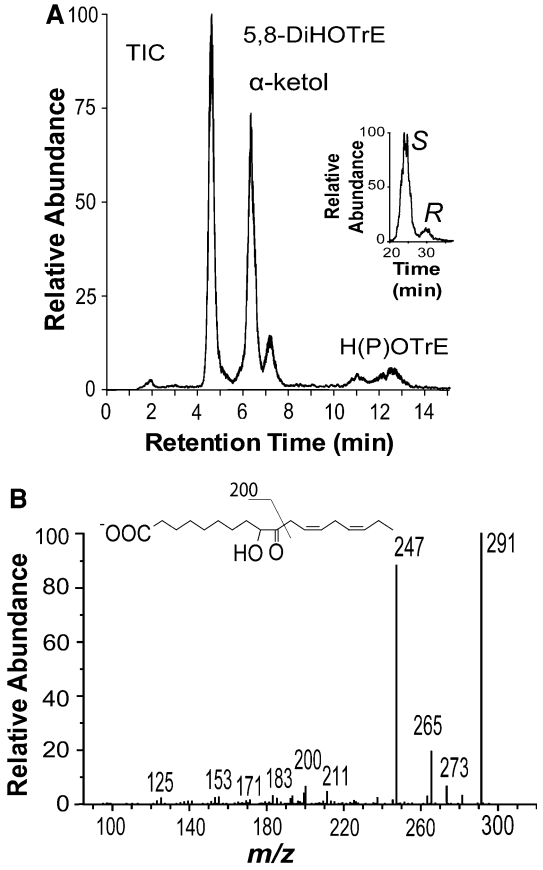

Fig. 2 LC–MS/MS analysis of oxidation of 18:3n-3 by subcellular fractions of *L. theobromae*. **a** RP-HPLC–MS analysis of metabolites formed by CBS 117454. TIC from MS/MS analysis of *m*/*z* 309 and 293. The two major peaks contained 5,8-DiHOTrE and an α-ketol, as indicated. Small amounts of H(p)OTrE were also detected. Strain CBS 122127 yielded a similar pattern. The inset chromatogram shows that the *S* and *R* stereoisomers of α-ketols were formed from hydrolysis of the allene oxide in a ∼10:1 ratio (CP-HPLC–MS/MS). **b** MS/MS spectrum (*m*/*z* 309 → full scan) of the α-ketol derived from hydrolysis of an allene oxide. The insert shows formation of the even numbered signal at *m*/*z* 200, which presumably is due to a radical anion [17]

16:3n-3 was oxidized to approximately equal amounts of 10-HHDTrE and 11-HHDTrE (Fig. 3). A steric analysis by CP-HPLC showed that 10*R*-HHDTrE and 11*S*-HHDTrE were mainly formed (Fig. 3b, c). It seems likely that the 10*R*- and 11*S*-DOX activities are due to the linolenate 9*R*-DOX activity. Chain shortening of 18:3n-3 by two carbons thus changed the oxygenation from C-9 to C-10, and led to biosynthesis 11*S*-HPHDTrE after hydrogen abstraction at C-9. 11*S*-HPHDTrE is a precursor of JA in plants [2], but it was not transformed by AOS of *L. theobromae*.

18:2n-6, *18:3n-6*, *and 20:2n-6* 18:2n-6 was transformed to a major polar metabolite, which eluted after 7 min during LC–MS/MS analysis (Fig. 4a). [U-^{13}C]Linoleic acid was oxidized in the same way. The mass spectrum of the main metabolite was identical with that of an α-ketol, 9-hydroxy-10-oxo-12Z-octadecenoic acid [17]. Small

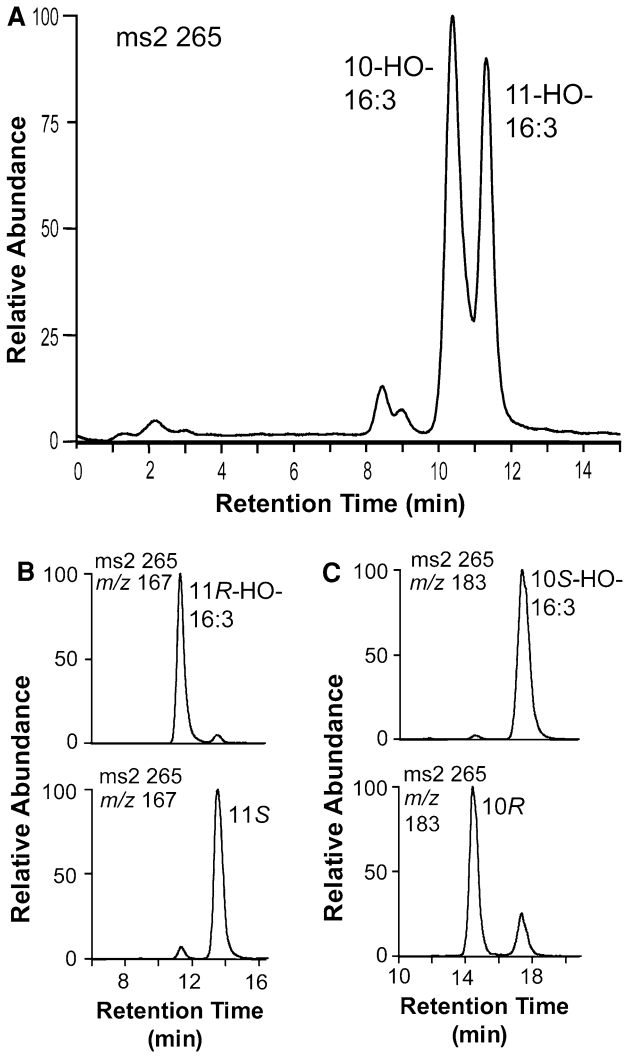

Fig. 3 Analysis of oxidation of 16:3n-3 by subcellular fractions of *L. theobromae*. The products formed by strain CBS 122127 were analyzed by RP- and CP-HPLC with ESI–MS/MS after reduction to alcohols with triphenylphosphine. **a** RP-HPLC. The first eluting major peak contained 10-HHDTrE (marked 10-HO-16:3) and the second peak II 11-HHDTrE (marked 11-HO-16:3). **b** CP-HPLC–MS analysis of 11-HHDTrE (*bottom chromatogram*) with aid of an 11*R*-HHDTrE standard (*top chromatogram*; the 11*R* stereoisomer was produced with Mn-LOX [32]). **c** CP-HPLC–MS analysis of 10-HHDTrE (*bottom chromatogram*) with aid of the 10*S* stereoisomer (prepared with Mn-LOX [32])

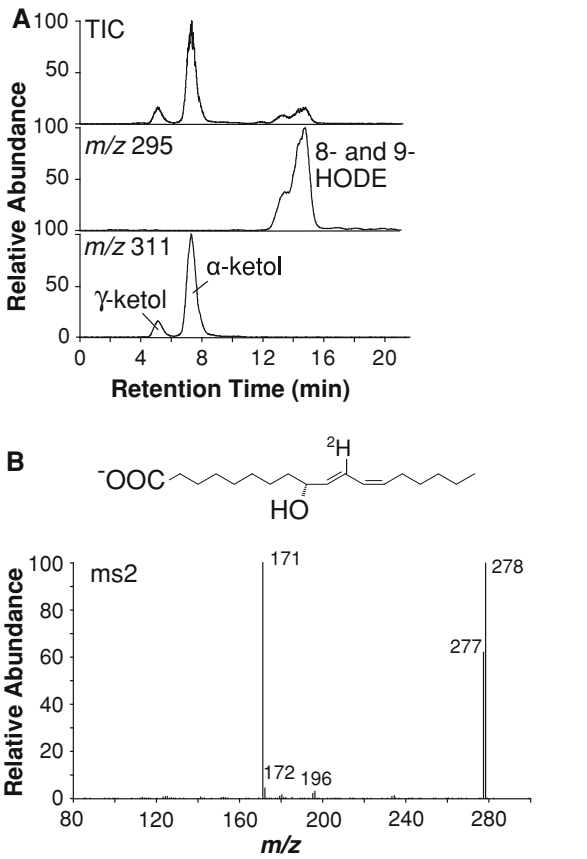

Fig. 4 LC–MS/MS analysis of oxidation of 18:2n-6 by subcellular fractions of *L. theobromae* (CBS 117454). **a** RP-HPLC–MS analysis showed that the major products were α- and γ-ketols and 8- and 9-HODE. **b** MS/MS analysis (*m/z* 292–298) → full scan) of 9-HODE from an incubation with [11*S*-^{2}H]18:2n-6 (95% ^{2}H). The latter was diluted with endogenous 18:2n-6 in the incubation. The signals at *m/z* 277 and 278 of this mass spectrum, mainly due to loss of water from the carboxyl group, showing that the deuterium label was retained, as *m/z* 277 and 278 were present in the same relative intensities as in 8-HODE of this experiment

amounts of the γ-ketol, 13-hydroxy-10-oxo-11*E*-octadecenoic acid, was detected after 5 min (Fig. 4a); MS/MS and MS/MS/MS spectra as reported [17].The two ketols were apparently formed from 9*R*-HPODE via hydrolysis of an unstable allene oxide, 9,10-epoxy-10,12*Z*-octadecadienoic acid [17]. Steric analysis of the α-ketol supported this mechanism of biosynthesis from 9*R*-HPODE, as CP-HPLC showed that it consisted mainly of the 9*S* stereoisomer (cf. insert in Fig. 2a). The 9*R*-DOX activity was present in the soluble fraction and the AOS activity in the microsomal fraction, which are in agreement with the distribution of plant LOX and AOS (CYP74) [2].

Other metabolites were 5,8-DiHODE, 8-H(P)ODE, and small amounts of 10-H(P)ODE and 13-H(P)ODE (~5% of 9-H(P)ODE). Steric analyses showed that 10-HODE was almost racemic (~60% *S*), whereas 13-HODE was mainly formed with *S* configuration (data not shown).

The oxidation of 18:2n-6 was investigated with [11*S*-^{2}H]18:2n-6 (95% ^{2}H). RP-HPLC with MS and MS/MS analysis of 9*R*-HODE showed that this deuterium label was retained (Fig. 4b), suggesting that the 9*R*-DOX of *L. theobromae* catalyzes suprafacial hydrogen abstraction at C-11 relative to the oxygenation at C-9. The apparent deuterium content, as judged from the ion intensities at *m/z* 277 and 288 of both 8- and 9-HODE, was 60%, due to dilution with endogenous 18:2n-6. The deuterium label was also retained in 13*S*-HODE, a minor metabolite in comparison with 9-HODE.

The 9*R*-DOX activity also transformed 18:3n-6 to 9-HPOTrE(n-6) as the main metabolite, but further transformation to an α-ketol could not be detected. This fatty acid was not oxygenated by 5,8-LDS in agreement with previous reports [31]. Significant formation of 13-HOTrE(n-6) was also detected, as judged from the signal intensities of characteristic ions [*m*/*z* 169 (80%; 9-HOTrE(n-6)] and *m*/*z* 193 [20%, 13-HOTrE(n-6)].

20:2n-6 was oxidized at C-11 and C-15, but these hydroperoxides were not further converted to α-ketols.

18:1n-9 and 18:1n-6 Oleic acid was exclusively oxidized by *L. theobromae* to 5,8-DiHOME and 8-H(P)OME, as shown in Fig. 5a. We could not detect signs of hydrogen abstraction at C-11, as judged from insignificant formation of 9-HOME(10*E*) and 11-HOME(9Z). This suggested that 18:1n-9 was not oxidized by the 9*R*-DOX activity.

The 9*R*-DOX activity might conceivably abstract the hydrogen at C-11 of 18:1n-6 with formation of 11*S*-HOME(12*Z*) or 13*S*-HOME(11*E*), whereas LDS enzymes mainly oxidize 18:1 with formation of stereoisomers with *R* configuration [25]. 13*S*-HOME(11*E*) was not detected. As shown in Fig. 5b, the formation of 11*S*-HOME(12*Z*) was only 10–15% of the biosynthesis of 11*R*-HOME(12*Z*) and could be attributed to oxidation by 5,8-LDS [24, 25].

In conclusion, 18:1n-9 and 18:1n-6 did not appear to be oxidized to a significant extent by the 9*R*-DOX activity.

13S-HPHTrE and *11S-HPHDTrE*—these hydroperoxides were not transformed by the AOS activity.

Discussion

Tsukada et al. [10] demonstrated that JA is formed in *L. theobromae* from 18:3n-3 [10]. In these experiments, the fungus was grown for 10 days with ^{13}C-labeled sodium acetate or with [$^{2}H_6$]18:3n-3, and found to secrete significant amounts of [^{13}C]JA, [$^{2}H_5$]JA, and [$^{2}H_5$]-iso-JA, respectively. These authors also isolated 12-OPDA (methyl ester), a key intermediate in the biosynthesis of JA in higher plants. Interestingly, NMR analysis of the deuterated 12-OPDA indicated a stereochemical difference between the fungal and plant cyclopentenone reductase reactions. This result suggested that fungal JA biosynthesis could have evolved independently from plant biosynthesis with both similarities and differences.

We did not find evidence for the presence in *L. theobromae* of the JA biosynthetic enzymes present in higher plants. Instead, we report that mycelia of two commercial strains of *L. theobromae* express prominent linolenate 9*R*-DOX and AOS activities after growth for about 10 days. These strains only secreted small amounts of JA, and we therefore also investigated the strain 2334 with large

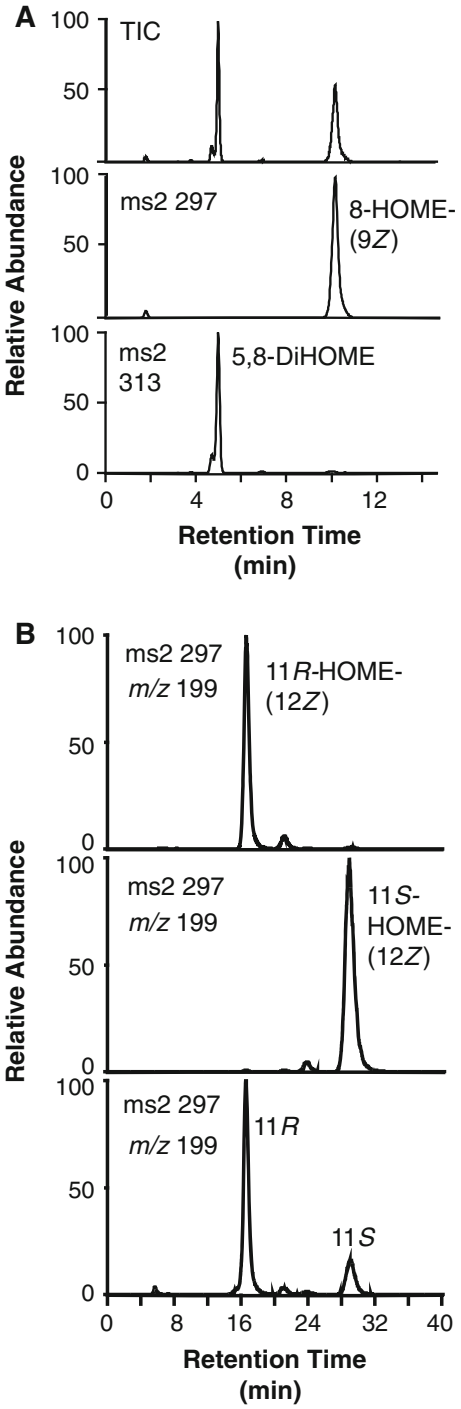

Fig. 5 LC–MS analysis of products formed during oxygenation of 18:1n-9 and 18:1n-6 by subcellular fractions of *L. theobromae*. **a** RP-HPLC analysis of oxidation of 18:1n-9 by strain CBS 122127. The two main products were 5,8-DiHOME and 8-HOME. **b** CP-HPLC analysis of oxidation of 18:1n-6–11-HOME(12*Z*) with aid of standards; the *top chromatogram* showing elution of 11*R*-HOME (prepared with Mn-LOX), the *middle chromatogram* elution of 11*S*-HOME (prepared with COX-1). The *bottom chromatogram* showing separation of products formed by *L. theobromae*. The latter thus mainly formed the 11*R* stereoisomer, and this metabolite can be attributed to the 5,8-LDS activity [25]

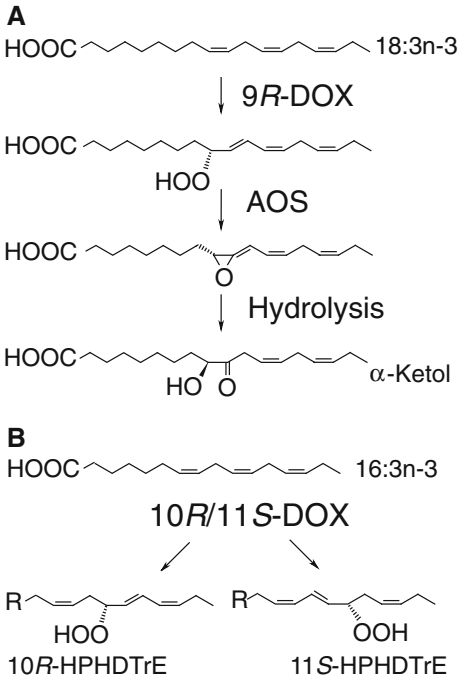

Fig. 6 Overview of the transformation of 18:3n-3 and 16:3n-3 to *cis–trans* conjugated hydroperoxides and an allene oxide by *L. theobromae*. **a** Oxidation of 18:3n-3. The soluble 9*R*-DOX activities are likely due to a lipoxygenase and the microsomal AOS activities to P450. **b** Oxidation of 16:3n-3. These metabolites are also formed by Mn-LOX but with the opposite chirality, and presumably formed by the same enzyme with oxidizes 18:2n-6 and 18:3n-3 to 9*R*-hydroperoxy metabolites

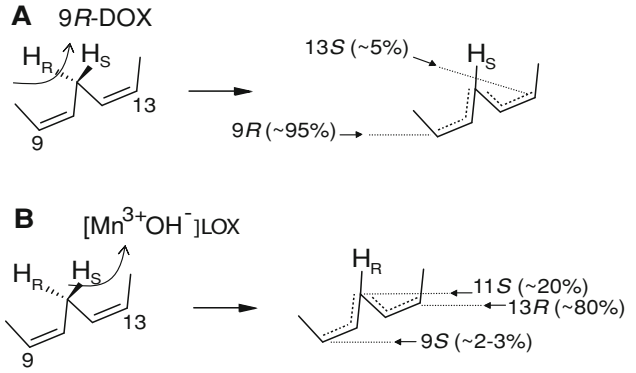

Fig. 7 A schematic presentation of the hydrogen abstraction mechanisms of 9*R*-DOX and Mn-LOX. **a** Mechanism of hydrogen abstraction of 18:2n-6 and direction of oxygenation by the 9*R*-DOX activity. **b** Mechanism of hydrogen abstraction of 18:2n-6 and direction of oxygenation by Mn-LOX. If the substrate could be oxidized by Mn-LOX in the reverse orientation, the products and the oxygenation mechanism would be similar to that of the 9*R*-DOX activity

capacity to secrete JA. This strain contained 18:3n-3 and secreted large amounts of JA at time points (10 and 12 days).

We found that 18:3n-3 and 18:2n-6 were both oxygenated to 9*R*-hydroperoxides, which were further transformed to allene oxides. The oxidation of 18:3n-3 by this pathway is summarized in Fig. 6a. The 9*R*-DOX activity showed broad substrate specificity, and also oxidized 16:3n-3, 18:3n-6, and 20:2n-6.

16:3n-3 can be converted to JA in plants, and the first step is biosynthesis of 11*S*-HPHDTrE [3]. This metabolite was formed by *L. theobromae*. 16:3n-3 was oxidized to approximately equal amounts of 10*R*-HHDTrE and 11*S*-HHDTrE (Fig. 6b), however, since we could not detect formation of α-ketols it is apparent that these hydroperoxides were not substrates of AOS. The oxidation of 16:3n-3 is summarized in Fig. 6b.

9*R*-DOX and AOS activities have also been reported in *A. terreus* [17]. The genome of *A. terreus* appears to lack LOXs, whereas there is no information of the genome of *L. theobromae*. At first, it seemed likely that these two fungi could form 9*R*-HPODE and allene oxides by homologous enzymes. The 9*R*-DOX activity was present in the soluble fraction, whereas the AOS activity was mainly present in microsomal fraction. Both fungi therefore likely express microsomal AOS of the cytochrome P450 family, but the 9*R*-DOX activities of *L. theobromae* and *A. terreus* could be due to different classes of dioxygenases, i.e., a heme-containing dioxygenase in case of *A. terreus* and a lipoxygenase in case of *L. theobromae*. The latter hypothesis is based on the following observations.

First, γ-linolenic acid (18:3n-6) was oxidized to 9-HPOTrE(n-6) as a prominent metabolite by *L. theobromae*. This fatty acid is readily oxidized by many plant LOX, but it is not oxidized by a series of fungal DOX-CYP fusion proteins, e.g., 5,8-LDS, 7,8-LDS, and 10*R*-DOX, and not by the 9*R*-DOX of *A. terreus* [17]. Second, LOXs oxygenate 18:1 only slowly [24], and we could not attribute the oxidation of 18:1n-6 or 18:1n-9 to the 9*R*-DOX activity of *L. theobromae*. Thirdly, we were struck by the similarities of Mn-LOX and 9*R*-DOX.

Both enzymes oxidize 16:3n-3 at the n-7 (C-10) and the n-6 carbons (C-11), albeit with different chirality [32]. The oxidation at the n-7 position is not a common feature of LOXs, but was recently reported by the LOX domain of a fusion protein of a cyanobacterium [33]. Oxygenation by 9*R*-DOX and Mn-LOX differ with regard to regiochemical and stereochemical specificity, but both enzymes catalyze suprafacial hydrogen abstraction and oxygenation [34], as outlined in Fig. 7. It is tempting to speculate that 9*R*-DOX activity of *L. theobromae* could be due to a LOX homologue of Mn-LOX with reverse substrate orientation in the active site (cf. Fig. 7). DOX-CYP fusions proteins and PGH synthases catalyze antarafacial hydrogen abstraction [24], whereas the 9*R*-DOX activity of *A. terreus* also is suprafacial [17]. The main difference between the two fungal 9*R*-DOX activities resides in the oxidation of 18:3n-3.

The AOS activity of *L. theobromae* appeared to be specific for 9*R*-HPODE and 9*R*-HPOTrE, since we could not detect significant formation of α-ketols from 16:3n-3, 18:3n-6, 20:2n-6, 13*S*- or 13*R*-HpOTrE. The substrate specificity of AOS of *A. terreus* is restricted to 9*R*-HPODE. In contrast, AOS (CYP74A) of plants are specific for 9*S*- or 13*S*-hydroperoxides [35, 36].

In summary, we report that *L. theobromae* efficiently oxidized 18:2n-6 and 18:3n-3 sequentially to 9*R*-hydroperoxides and to unstable allene oxides. The AOS activity is microsomal and probably catalyzed by a homologue of CYP74, whereas the soluble 9*R*-DOX activity might be due to a LOX. It appears that the genome sequence of *L. theobromae* will be needed to elucidate the detailed mechanism of JA formation in this fungus.

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