Three putative oxylipin biosynthetic genes integrate sexual and asexual development in Aspergillus nidulans

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Oxylipins called psi factors have been shown to alter the ratio of asexual to sexual sporulation in the filamentous fungus Aspergillus nidulans. Analysis of the A. nidulans genome has led to the identification of three fatty acid oxygenases (PpoA, PpoB and PpoC) predicted to produce psi factors. Here, it is reported that deletion of ppoB (ΔppoB) reduced production of the oleic-acid-derived oxylipin psiB and increased the ratio of asexual to sexual spore development. Generation of the triple mutant ΔppoAΔppoBΔppoC resulted in a strain deficient in producing oleic- and linoleic-acid-derived 8′-hydroxy psi factor and caused increased and mis-scheduled activation of sexual development. Changes in asexual to sexual spore development were positively correlated to alterations in the expression of brlA and veA, respectively. PpoB and/or its products antagonistically mediate the expression levels of ppoA and ppoC, thus revealing regulatory feedback loops among these three genes. Phylogenetic analyses showed that ppo genes are present in both saprophytic and pathogenic Ascomycetes and Basidiomycetes, suggesting a conserved role for Ppo enzymes in the life cycle of fungi.

INTRODUCTION

Oxylipins compose a family of structurally related oxygenated long-chain fatty-acid-derived molecules that exhibit crucial biological activities as signals of intra- and intercellular communication in animals, plants and fungi (Herman, 1998; Noverr et al., 2003). These molecules provide a number of functions ranging from regulation of reproduction in invertebrates to formation of innate defence mechanisms in plants (Blee, 2002; Farmer et al., 2003; Funk, 2001; Howe & Schilmiller, 2002; Noverr et al., 2003). Oxylipin production is ubiquitous among pathogenic and saprophytic fungi and appears to play a role in life cycle control particularly in sexual and asexual development (Herman, 1998; Kock et al., 2003; Noverr et al., 2003; Strauss et al., 2000). For instance, in various members of Mucorales, immunofluorescence microscopy showed that 3-OH oxylipins are associated with asexual reproductive structures (e.g. sporangium, columnella and aggregating sporangiospores), and in the yeast Dipodascopsis uninucleata with the sexual reproductive phase of the life cycle (e.g. gametangia, ascii and matrix of released aggregating ascospores) (Kock et al., 1998, 2003; Strauss et al., 2000).

In Aspergillus nidulans, a model system for fungal development, endogenous oleic- and linoleic-acid-derived oxylipins, collectively named ‘psi factor’ (precocious sexual inducer), influence the development of cleistothecia, reproductive bodies containing the sexual ascospores, and conidiophores, sporophores bearing the asexual conidiospores (Calvo et al., 1999, 2001, 2002; Champe & el-Zayat, 1989; Tsitsigiannis et al., 2004b). Psi factor is primarily a mixture of secreted hydroxylated oleic (18:1) and linoleic (18:2) molecules termed psiβ and psiα, respectively (Champe et al., 1987; Champe & el-Zayat, 1989). The positioning of the hydroxy groups on the fatty acid backbone further designates the psi compounds as psiB (8′-hydroxy-), psiA (5′,8′-dihydroxy-) and psiC (designating a lactone ring at the 5′ position of psiA) (Mazur et al., 1990, 1991). Champe and coworkers discovered that purified psiBz and psiCz stimulate sexual and inhibited asexual spore development (Champe et al., 1987; Champe & el-Zayat, 1989). PsiAz, however, enhanced asexual sporulation leading to the postulation that the ratio of psiAz to psiBz and psiCz determines whether asexual or sexual sporulation dominates (Champe et al., 1987; Champe & el-Zayat, 1989).
A role for oxylipins in Aspergillus development was supported by further studies demonstrating that purified linoleic acid and plant hydroperoxy linoleic acids exhibit sporogenic activities toward several Aspergillus spp., including A. nidulans and the seed-infecting fungi Aspergillus flavus and Aspergillus parasiticus (Calvo et al., 1999). In all of these species, the primary effect of linoleic acid and hydroperoxy linoleic acids was to induce precocious and increased conidial development. This response, however, was concentration-dependent as lower amounts of linoleic acid and 9S-hydroperoxy (9S-HPODE) linoleic acid stimulated sexual spore development rather than conidial development in A. nidulans (Calvo et al., 1999).

Efforts to elucidate the oxylipin biosynthetic pathway in A. nidulans have resulted in the characterization of two genes, ppoA and ppoC, that encode putative fatty acid oxygenases required for biosynthesis of the linoleic-acid-derived psi and the oleic-acid-derived psiβ (Tsitsigiannis et al., 2004a) respectively. These genetic studies support the observations that oxylipin production is important in regulating asexual and sexual sporulation and have led to the hypothesis of the existence of a fungal ‘oxylipin signature-profile’ that plays an important role in integrating mitotic and meiotic spore balance (Tsitsigiannis et al., 2004a).

The goal of this study was to characterize a third oxylipin biosynthetic gene, ppoB, found in A. nidulans. PpoB contributes to the formation of the oleic-acid-derived oxylipin psiBI and exhibits a regulatory role in fungal sporulation. Deletion of ppoB significantly increased the ratio of asexual to sexual spore development by eightfold. This was in contrast to the previously characterized AppoC and ΔppoAΔppoC strains where sexual spore development was significantly elevated (Tsitsigiannis et al., 2004a). Creation of the triple mutant ΔppoAΔppoBΔppoC yielded an ascospore-overproducing phenotype crippled in the ability to produce oleic- and linoleic-acid-derived psiB. Alterations in the ratio of asexual to sexual spore production were concomitantly reflected in mRNA levels of a transcription factor required for conidial (brlA) development and the velvet gene (veA) required for ascospore development in A. nidulans. This study integrates the characterization of the three oxylipin biosynthetic genes found in A. nidulans, demonstrating their significance in regulation of the fungal reproductive cycles. The existence of Ppo orthologues in all species of filamentous fungi found in the available genomic databases may reflect a conserved function of these enzymes in the life cycle of fungi.

METHODS

Fungal strains, growth conditions and genetic manipulations. All A. nidulans strains used in this study (Table 1) were maintained on defined glucose minimal medium (GMM) (Calvo et al., 2001) with appropriate supplements as needed at 37°C in continuous dark or white light. Sexual crosses and protoplast transformation of A. nidulans strains were conducted according to standard techniques (Pontecorvo et al., 1953; Yelton et al., 1984). Illumination was carried out in an incubator equipped with General Electric 15 W broad-spectrum fluorescent light bulbs (F15T12CW) placed 50 cm above the plates. RNA was extracted from grown strains by inculcating 30 ml liquid GMM with 1 x 10⁶ spores ml⁻¹ of the appropriate strain before incubating for 24 or 72 h (stationary conditions) prior to harvesting. Radial, vegetative growth and germination tests were performed in triplicate as described previously (Tsitsigiannis et al., 2004a). Microscopic observations were conducted using an Olympus BX60F-3 microscope and an Olympus SZ-60 stereo and images were captured by an Olympus digital camera.

Nucleic acid manipulations. Construction, maintenance and isolation of recombinant plasmids were performed using standard techniques (Sambrook & Russell, 2001). Fungal chromosomal DNA was extracted from lyophilized mycelia using previously described techniques (Lee & Taylor, 1990). Total RNA was extracted from lyophilized mycelia using TRizol reagent (Invitrogen) according to manufacturer’s recommendations. Approximately 20 μg total RNA was used for Northern analysis using a 1-2 % agarose/1-5 % formaldehyde gel transferred to Hybond-XL membrane (Amersham Pharmacia Biotech). The PCR product obtained with primers ppoB-F17 (5’-GGCGTTGCTGATTAGG-3’) and ppoB-F4 (5’-ACTCAACACGCTTCAACTC-3’) using the cosmid pLFM13 as template was used as ppoB-specific DNA probe for Southern and Northern hybridizations. Gene expression studies were performed with appropriate probes: a 4.5 k b SalI brlA-specific fragment from pTA111 (Adams et al., 1988), a 1.1 k b nsdD-specific PCR product obtained with nsdD-5’ and nsdD-3’ (Tsitsigiannis et al., 2004a), a 4 k b ppoA-specific PCR product obtained with primers ppoA-F2 and ppoA-R2 (Tsitsigiannis et al., 2004b), a 4.1 k b ppoC-specific PCR product obtained with primers ppoC-F16 and ppoC-R18 (Tsitsigiannis et al., 2004a) and a 1.1 k b veA-specific PCR product obtained with veA-5’ (5’-TTTTGTGTATCCCATCAGATT-3’) and veA-3’ (5’-GTGAGCAGAAGCAGGTGAGG-3’) (Kim et al., 2002). Detection of signals was carried out with a Phosphorimager SI (Molecular Dynamics). Densitometry data were obtained and analysed with PDQuest software (Bio-Rad). Nucleotide sequences were analysed and compared using Sequencer (Gene Codes) and ClustalW (www.ebi.ac.uk/clustalw/) programs (Chenna et al., 2003).

Molecular cloning, disruption and complementation of the A. nidulans ppoB gene. The ppoB gene was identified by a BLASTn search of the Cereon (Monsanto Microbial Sequence Database: www.cereon.com) and Broad Institute A. nidulans databases (www.broad.mit.edu/annotation/fungi/aspergillus) based on the amino acid sequence of linoleate diol synthase (Lds) cloned from Geuannamomyces graminis that was used as query sequence (Hornstein et al., 1999). Oligonucleotides ppoB-F1 (5’-AGTAGGCCG-TGGCGGAGGTTG-3’) and ppoB-R1 (5’-AAGCGAGGAGTGG- GGTTTG-3’) were designed based on the obtained contig ANI61C10915, predicting a fragment with high identity to Lds and PpoA specific fragment from pTA111 (Adams et al., 1988), a 1.1 k b nsdD-specific PCR product obtained with nsdD-5’ and nsdD-3’ (Tsitsigiannis et al., 2004a), a 4 k b ppoA-specific PCR product obtained with primers ppoA-F2 and ppoA-R2 (Tsitsigiannis et al., 2004b), a 4.1 k b ppoC-specific PCR product obtained with primers ppoC-F16 and ppoC-R18 (Tsitsigiannis et al., 2004a) and a 1.1 k b veA-specific PCR product obtained with veA-5’ (5’-TTTTGTGTATCCCATCAGATT-3’) and veA-3’ (5’-GTGAGCAGAAGCAGGTGAGG-3’) (Kim et al., 2002). Detection of signals was carried out with a Phosphorimager SI (Molecular Dynamics). Densitometry data were obtained and analysed with PDQuest software (Bio-Rad). Nucleotide sequences were analysed and compared using Sequencer (Gene Codes) and ClustalW (www.ebi.ac.uk/clustalw/) programs (Chenna et al., 2003).
gene and ppoB flanking sequences, was constructed using the following methodology. First, the modified primer pairs ppoB-3DF1-Hin-cassette (5'-GTCAGCTGGTGATCGACGGGTTACCGGC-3'), which is 33 bp downstream of the predicted start codon of ppoB (the ppoB ORF is in an inverted position in A. nidulans genomic DNA), and ppoB-3DR1-Pst (5'-CCAGTTACTTTCCACGAAGCCTGG-3'), were used to PCR-amplify a 1.2 kb flanking region at the 5' UTR of the ppoB ORF using cosmid pLM13 as template. The resulting amplified BamHI–KpnI PCR fragment was subcloned into p14 harboring the pLFM13 as template. The resulting amplified ppoB::ppoC::metG veA trpC801 hybrid vector was used to transform A. nidulans trpC gene, which can reconstruct the trpC801 mutation by single crossing over. TDIT55.7 to give the complemented ppoB::ppoC prototroph RDIT91.7. RDIT30.32 and FGSC33, RDIT30.35 and RDIT30.37, and RDIT30.35 and RDIT30.37 are progeny of the cross between FGSC237 and RDIT1.1. RDIT54.13 was derived from the cross between TTMK1.97 and RTMK2.60. RDIT54.13 from the cross between RDIT30.32 and FGSC33, RDIT35.37 from the cross between RDIT45.25 and RAMC22.1, and RDIT45.25 from the cross between RDIT30.35 and FGSC33. RDIT30.32 and RDIT30.35 are progeny of the cross between FGSC237 and RDIT1.1. RDIT54.13 was derived from the cross between TTMK1.97 and RTMK2.60.

Complementation of the original ΔppoB transformant TTMK2.60 was achieved using the vector pBJK1.6. Plasmid pBJK1.6 was created by inserting the 6.5 kb KpnI–BamHI fragment from plasmid pTMK1.4, containing the predicted promoter, the coding sequence and the termination cassette of ppoB, into pSH96 (Wieser & Adams, 1995). pSH96 harbours a 1.8 kb fragment of the 5' portion of the A. nidulans trpC gene, which can reconstruct the trpC801 mutation by single crossing over. TDIT10.5 was one of the tryptophan prototrophs containing the ppoB::trpC allele. TDIT10.5 was further crossed with RDIT55.7 to give the complemented ΔppoB prototroph RDIT91.7.

Fatty acid analysis. Strains were grown on 15 ml liquid GMM in Petri dishes under stationary conditions at 37 °C in the dark. Mycelial mats were collected after 72 h, lyophilized, weighed and homogenized mechanically using an Ultra-Turax T25 disperser (Ika Werke). Lipids were extracted and converted into fatty acid methyl esters (FAME) derivatives using 2% sulfuric acid in methanol as described by Browse et al. (1986). To convert hydroxylated FAMEs into corresponding trimethylsilyl ether (TMSi) derivatives, the methanol phase was removed in vacuo and the remaining residue was dissolved in 80 µl of a mixture of N,O-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (29:1, by vol.) (Sylon BFT Kit; Supelco) (Zarnowski et al., 2000). The reaction was incubated at 90 °C for 30 min and TMSi-FAMEs were recovered in a small volume of hexane. Both FAMES and hydroxylated FAMEs were separated by GC (Thermoquest Trace GC) on an
RTX-5MS 0·25 μm fused silica column (Restek) and identified by MS on an inline Finnigan Polaris mass spectrometer. One microlitre of the sample was analysed by GC equipment programmed as follow: 80 °C (held for 2 min) increased at 20 °C min⁻¹ up to 220 °C, 30 °C min⁻¹ to 300 °C and then held at 300 °C for 2 min. The injector temperature was 300 °C and helium (1 ml min⁻¹, constant flow) was used as a carrier gas. For MS, electron impact mode was used and the ion source was 280 °C. The electron energy was 70 eV, ionization current 100 μA and the scan speed was 0·6 s per decade. Scans were recorded in a range from 35 to 600 amu. Fatty acids were identified by comparison of retention times with a set of authentic fatty acids standards, whereas hydroxylated derivatives of fatty acids were identified by MS on the basis of their fragmentation patterns reported by Calvo et al. (2001) and Fox et al. (2000).

**Physiological studies.** All strains used for physiological studies were prototrophic and carried the wild-type allele veA (Champe et al., 1994; Kim et al., 2002). Asexual and sexual spore production studies were carried out on plates containing 30 ml solid 1·5% GMM. For each plate a 5 ml top layer of cool melted 0·7% agar-GMM containing 10⁶ conidia of the appropriate strain was added. Cultures were incubated in continuous dark or light at 37 °C since it is known that sporulation in *A. nidulans* wild-type strains carrying the veA locus is influenced by the light or dark regime (light induces asexual and delays and reduces sexual spore production) (Champe et al., 1994; Kim et al., 2002). A core of 12·5 mm diameter was removed from each plate at the appropriate time interval and homogenized for 1 min in 3 ml sterile water supplemented with 0·01% Tween 80 to facilitate the release of the hydrophobic spores. Conidia and ascospores were counted using a haemocytometer. The experiments were performed with four replicates. Spore data were statistically compared by analysis of variance (ANOVA) and Fisher’s Least Significant Difference (LSD) using the Statistical Analysis System (SAS Institute, Cary, NC, USA).

**Phylogenetic analysis.** To examine the conservation of *ppo* genes across the Ascomycetes and Basidiomycetes we searched the following genomic databases for *ppo* homologues: *A. nidulans* (www.broad.mit.edu/annotation/fungi/aspergillus), *Aspergillus fumigatus* (www.tigr.org/db/tdb/c2ki/afu1), *Neurospora crassa* (www.broad.mit.edu/annotation/fungi/neurospora), *Fusarium graminearum* (www.broad.mit.edu/annotation/fungi/fusarium), *Fusarium verticillioides* (www.tigr.org/db/tgi/cw/cwgi2), *Fusarium sporotrichioides* (www.genome.ou.edu/psoro.html), *Magnaporthe grisea* (www.broad.mit.edu/annotation/fungi/magnaporthe), *Phaeoacremonium strictum* (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html), *Ustilago maydis* (www.broad.mit.edu/annotation/fungi/ustilago_maydis/), *Coprinus cinereus* (www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), *Cryptococcus neoformans* (www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans/), *Histoplasma capsulatum* (www.genome.wustl.edu/blast/histo_client.cgi). *Candida albicans* (http://sequence-www.stanford.edu/group/candida/index.html), *Saccharomyces cerevisiae* (www.yeastgenome.org) and *Schizosaccharomyces pombe* (www.genedb.org/genedb/pombe/index.jsp). The amino acid sequences of the oxylipin-producing linoelate diol synthase (*lds*) gene from *G. graminis* var. *graminis* and the *A. nidulans* *ppoA*, *ppoB* and *ppoC* genes were utilized as the initial queries in our search. BLASTP hits of e⁻⁴⁰ or lower were aligned and a phylogenetic tree was created using the ClustalW program. Pairwise scores between the amino acid sequences were calculated as the number of identities in the best alignment divided by the number of residues compared (gap positions are excluded). A guide tree was calculated based on the distance matrix that was generated from the pairwise scores. The phylogenetic tree was calculated based on the multiple alignment and the distances between the amino acid sequences in the alignment were then used by the neighbour-joining method (PHYLIP) (Chenna et al., 2003) to make the tree shown in Fig. 6. The sequences of other oxylipin biosynthetic enzymes such as fungal lipoxygenases, mammalian prostaglandin synthases or cylooxygenases (GenBank accession numbers: human PGH2, NP_000954; mouse PGH2, 5COX_A; horse PGH2, 019183) and a tobacco pathogen-induced 9-oxygenase (PIOX, T03631) were also used in this analysis. The *A. nidulans* polyketide synthase gene (*stcA*) was the outgroup sequence used to root our phylogenetic analysis.

**RESULTS**

**Analysis of the *A. nidulans* ppoB gene**

BLAST searches of the *A. nidulans* publicly available genome databases with the oxylipin-producing linoelate diol synthase (*lds*) gene from the filamentous fungus *G. graminis* var. *graminis* (Hornsten et al., 1999) revealed the presence of three genes named *ppoA*, *ppoB* and *ppoC*. Disruption of *ppoA* (Tsitsigiannis et al., 2004b) and *ppoC* (Tsitsigiannis et al., 2004a) in *A. nidulans* led to strains defective in producing monohydroxy linoelate and oleic psi factor. In this study we characterized the role of *ppoB* (GenBank accession no. AY940146). The gene is located in chromosome III and, based on the predicted annotation by the Broad Institute, PpoB encodes a 1019 aa sequence after an 11-intron splicing event of the genomic DNA. The size of the mRNA transcript was confirmed by Northern analysis (data not shown). Protein domain searches against the Pfam database (http://pfam.wustl.edu) indicated PpoB residues 143–477 have domains similar to animal haem peroxidases (1 × 10⁻¹¹) and residues 849–976 have domains similar to cytochrome P450 oxygenases (4 × 10⁻⁹). Comparative sequence analysis (ClustalW) between the amino acid sequences of PpoB and the previously characterized oxygenases PpoA and PpoC showed 38 and 35% identity respectively. PpoB also shares similarity with the *Magnaporthe grisea* linoelate diol synthase, the SpS1 protein from *Ustilago maydis* and various predicted proteins from existing filamentous fungal databases as described below. Finally, PpoB contained the putative hydrophobic subdomain known as a ‘proline knot’ that is characteristic for targeting plant proteins to lipid bodies (Abell et al., 1997; Chen & Tzen, 2001). PpoA and SpS1 also contain the proline knot motif and are known to be localized to lipid bodies (Huber et al., 2002; Tsitsigiannis et al., 2004b).

**Phenotypic characterization of the *A. nidulans* ΔppoB mutant**

To functionally characterize the role of PpoB in fungal development, a *ppoB* null mutant (Δ*ppoB*) was created by homologous recombination. PCR and Southern analysis of 67 transformants revealed the replacement of the wild-type *ppoB* gene with the *pyroA* marker gene in two transformants that showed identical phenotypes (data not shown). Transformant TTMK2.60 was selected and crossed to produce a prototrophic Δ*ppoB* strain (RDT59.1) which was used for further physiological and molecular analyses. Both Δ*ppoB* and the triple Δ*ppoA*Δ*ppoB*Δ*ppoC* mutant had
Table 2. Psi factor composition of mycelia of A. nidulans oxylipin mutants

The analysis was carried out with 72 h-old mycelia grown in liquid GMM under stationary conditions at 37 °C in the dark. Values are the means of three replications ± SE. Statistical analysis was performed by using Student’s t-test and significance to wild-type oxylipin composition is indicated as follows: *, P < 0.001.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hydroxy-FAME [μg (g mycelium dry wt)⁻¹]</th>
<th>psiBβ (8-HOE†)</th>
<th>psiBα (8-HODE†)</th>
</tr>
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<tbody>
<tr>
<td>Wild-type†</td>
<td>5.87 ± 0.70</td>
<td>2.19 ± 0.87</td>
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</tr>
<tr>
<td>ΔppoB</td>
<td>2.66 ± 0.08*</td>
<td>1.93 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>ΔppoAΔppoBΔppoC</td>
<td>0.11 ± 0.11*</td>
<td>0.35 ± 0.33*</td>
<td></td>
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</tbody>
</table>

†psiBβ, 8-HOE (8-hydroxy oleic acid); psiBα, 8-HODE (8-hydroxy linoleic acid).
‡Wild-type values were taken with permission from Tsitsiannis et al. (2004b). The psi analysis was performed at the same time for all the Δppo mutants.

no alterations in radial growth on solid GMM, or vegetative development in liquid GMM, or spore germination compared to wild-type (data not shown); however, both asexual and sexual development were altered as described below. Complementation of the ΔppoB strain with the ORF of ppoB driven by its predicted native promoter returned the wild-type phenotype, thus confirming that the effects on sexual and asexual sporulation described below were solely due to the deletion of ppoB (data not shown).

Fatty acid and oxylipin analysis

To investigate the role of PpoB as a putative fatty acid oxygenase, oxylipin and fatty acid composition was assessed in both the ΔppoB and the triple mutants. GC-MS analysis of the two most abundant psi factor components, the oleic-acid-derived psiBβ [8-HOE or 8-hydroxy-9(Z)-octadecenoic acid] and the linoleic-acid-derived psiBα [8-HODE or 8-hydroxy-9(Z),12(Z)-octadecadienoic acid] led to the conclusion that deletion of the ppoB allele resulted in a reduction of the oleic-acid-derived psiBβ molecule (Table 2). The triple mutant was deficient in producing either oleic- or linoleic-acid-derived psiB factors (Table 2), suggesting that the three Ppo proteins are implicated in psiB factor formation derived from oleic and linoleic acid. The presence of linoleic- or oleic-acid-derived psiA or psiC was not detected in any samples in accordance with previous studies (Calvo et al., 2001).

Mycelial fatty acid content of the fatty acid oxygenase mutants was assessed using GC analysis from mycelia grown under dark conditions at 37 °C. Table 3 shows the percentage of fatty acids produced by the wild-type, ΔppoB and ΔppoAΔppoBΔppoC strains as well as the proportion of the most prevalent fatty acids detected in the FAME mixture. Deletion of ppoB did not alter the total fatty acid percentage per gram mycelium compared to wild-type. However, the triple mutant showed a 50% reduction in the amount of total fatty acids produced compared to wild-type. Furthermore, both ΔppoB and the triple mutant showed an increase in saturated fatty acids and a decrease in unsaturated fatty acids. This was especially notable in the triple mutant where the wild-type saturated/unsaturated fatty acid ratio of approximately 1:1 was shifted to 3:1 in this mutant.

PpoB acts as regulator of spore development

Conidia and ascospore production was assessed on GMM under light and dark conditions at 37 °C. Under both conditions ΔppoB produced significantly more conidia (2- to 3-fold increase depending on light or dark regime), but fewer ascospores (2- to 5-fold decrease) than the wild-type (P < 0.001) (Fig. 1 and Fig. 2). These results were maintained over a time period of 10 days (data not shown). Overall, the ratio of conidia to ascospores increased approximately eightfold in the ΔppoB mutant after 6 days cultivation in dark.

Deletion of ppo genes led to increased and mis-scheduled activation of sexual development in A. nidulans

We next examined the phenotype of the ΔppoAΔppoBΔppoC mutant which contained only trace amounts of psiB

Table 3. Fatty acid composition of mycelia of A. nidulans oxylipin mutants

The analysis was carried out with 72 h-old mycelia grown in liquid GMM under stationary conditions at 37 °C in the dark. Values are the means of three replications ± SE.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Weight percentage of major FAMEs†</th>
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<tbody>
<tr>
<td></td>
<td>Palmitic acid (16:0)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>31.10 ± 0.85</td>
</tr>
<tr>
<td>ΔppoB</td>
<td>44.01 ± 1.05</td>
</tr>
<tr>
<td>ΔppoABC‡</td>
<td>62.79 ± 2.75</td>
</tr>
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†Weight percentage FAME based on lyophilized weight of mycelia.
‡ΔppoABC = ΔppoAΔppoBΔppoC.
**Fig. 1.** Deletion of *ppoB* increases conidial production and decreases ascospore production. Cultures of wild-type (RDIT9.32; white bars) and Δ*ppoB* (RDIT59.1; grey bars) were grown for 2, 4 and 6 days at 37 °C under dark and light conditions on GMM plates. (a) Conidia production of cultures grown in the dark; (b) conidia production of cultures grown in the light; (c) ascospore production of cultures grown in the dark; (d) ascospore production of cultures grown in the light. Values are the mean of four replicates and error bars represent standard errors. Columns with an asterisk represent values for the same day that differ significantly from control (*P*<0.001).

**Fig. 2.** *ppo* genes are essential for balancing conidiophore/cleistothecia formation. Cultures of *A. nidulans* wild-type (RDIT9.32; WT) (a, d), Δ*ppoB* (RDIT59.1) (b, e) and Δ*ppoAΔppoBΔppoC* (RDIT62.3; Δ*ppoABC*) (c, f) were grown at 37 °C on solid GMM. Induction of the asexual and suppression of the sexual fruiting bodies was observed in the Δ*ppoB* mutant. The opposite is observed in the triple mutant. Each strain was inoculated with 10⁶ conidia per plate and cultures were grown for 8 days under dark (a–c) under light (d–f) conditions. Black balls are cleistothecia (Cl) and smaller green spheres are conidiophore heads.
molecules. The triple mutant led to precocious development of Hülle cells (multinucleate globular cells that surround and probably feed the cleistothecium) 2 days after inoculation on agar medium and to the production of a large number of cleistothecia (Fig. 2). Furthermore, the triple mutant was able to produce Hülle cells and cleistothecia in GMM liquid cultures in contrast to wild-type, which was unable to form any sexual structures under these conditions (data not shown). In radial growth experiments the triple mutant showed approximately 3 mm retardation (16% decrease compared to wild-type) of the mature conidiophore zone (fully developed conidiophores) and a 16–24 h delay in conidiophore development (data not shown). Vegetative hyphal growth at both the colony level on solid media (GMM) and fungal biomass in liquid cultures (GMM) remained unaltered. Analytical spore counts demonstrated that the ΔppoAΔppoBΔppoC strain produced fewer conidia and more ascospores under both light and dark conditions (Fig. 3). The ratio of asexual spore development to sexual spore development under dark conditions decreased approximately 22-fold in the triple mutant compared to wild-type.

Changes in meiotic/mitotic spore ratio are correlated with transcriptional alterations in brlA and veA expression

To gain further insight into the mechanism through which Ppo proteins and/or their products govern morphological differentiation in A. nidulans, we analysed the role of ppo mutations in regulation of the two key sporulation transcription factors: BrlA, the major mediator of asexual development (Adams et al., 1988) and NsdD, one regulator of sexual development (Han et al., 2001), and VeA, another major regulatory protein of sexual differentiation (Kim et al., 2002). We were interested to determine whether the abnormal activation of asexual or sexual development in ΔppoB and ΔppoAΔppoBΔppoC was correlated with brlA, nsdD and/or veA expression. As shown in Fig. 4, brlA transcripts were upregulated in the ΔppoB strain and downregulated in the triple mutant strain. These transcriptional alterations positively correlated with the relative increase (ΔppoB) and decrease (ΔppoAΔppoBΔppoC) in conidial production. Expression analysis of the nsdD gene showed that the transcript was slightly upregulated in the ascospore-overproducing strain ΔppoAΔppoBΔppoC and slightly downregulated in the ΔppoB mutant. Interestingly, transcriptional analysis of the veA gene demonstrated that is significantly upregulated in ΔppoAΔppoBΔppoC at 48 h (Fig. 4), a time point that coincides with the initiation of Hülle cell production. veA was expressed at similar levels to wild-type at the 72 h time point.

ppoA and ppoC are oppositely regulated in the ΔppoB strain

To characterize a potential regulatory role of PpoB in the transcriptional control of the ppo gene family, expression studies were conducted analysing the mRNA levels of ppoA and ppoC in the ΔppoB mutant. As shown in Fig. 5, ppoA is downregulated and ppoC upregulated when PpoB is not present. However, the ppoB transcript, which was observed at very low levels in wild-type, was not expressed at detectable levels in ΔppoA or ΔppoC mutants (data not shown). These data indicate that PpoB and/or its products antagonistically mediate the expression levels of ppoA and ppoC and uncover a regulatory relationship between these three proteins and/or their enzymic products that affect the balance of ascospore and conidia production.

![Figure 3](http://mic.sgmjournals.org/fig3.png)

**Fig. 3.** ΔppoAΔppoBΔppoC (RDIT62.3; black bars) shows decreased conidia and increased ascospore production compared to wild-type (RDIT9.32; white bars) under both dark and light conditions (P<0.001). Cultures of A. nidulans wild-type and ΔppoAΔppoBΔppoC were grown at 37 °C under dark and light conditions in GMM. Conidia (a) and ascospore (b) production of 6-day-old cultures grown in dark or light is shown. Values are the mean of four replicates and error bars represent standard error. Columns with asterisks (*) represent values for the same day that differ significantly from the wild-type (P<0.001).
In silico identification and comparison of fungal oxylipin biosynthetic genes

Considering the effects of ppo deletions on A. nidulans development coupled with the extent of literature implicating oxylipins in fungal differentiation processes (Fox et al., 2000; Kock et al., 2003; Noverr et al., 2003; Strauss et al., 2000), we examined the known fungal databases for evidence of putative Ppo orthologues as a first step in exploring a widespread role for oxylipins in fungal development. Extended combinatorial BLASTP and TBLASTN searches with Ppo, mammalian and plant fatty acid oxygenases revealed high similarities to hypothetical proteins from filamentous and dimorphic fungi, but not from Cryptococcus neoformans, Candida albicans, Saccharomyces cerevisiae and Schizosaccharomyces pombe. Our phylogenetic analyses suggested that ppo genes are conserved in both saprophytic and pathogenic Ascomycetes and Basidiomycetes (Fig. 6). The search of the final entire genomic databases of filamentous fungi revealed that A. fumigatus, like A. nidulans, contains three ppo genes, Aspergillus oryzae and Fusarium graminearum have five and four respectively, and N. crassa and Magnaporthe grisea contain two ppo genes.

DISCUSSION

Aspergillus is a genus of significant agricultural, medical and industrial importance. For Aspergillus species that are
opportunistic pathogens of plants and animals, spores serve as the major source of primary and secondary inoculum and are important factors in microbial colonization, dissemination and pathogenesis (Agrios, 1997; Alexopoulos et al., 1996). As studies have suggested that seed- and fungus-derived oxylipins act as sporogenic and developmental factors in these species (Calvo et al., 1999), our recent studies have been directed towards the identification of the genes required for formation of these fatty acids in both Aspergillus spp. and host seed crops (Burow et al., 2000; Calvo et al., 1999, 2001, 2002; Tsitsigiannis et al., 2002, 2004a, b; Wilson et al., 2004). The experiments presented here integrate the characterization of three A. nidulans genes encoding putative oxylipin biosynthetic enzymes and provide evidence that they are involved in coordinating meiospore/mitospore balance.

**Fig. 6.** Phylogenetic tree of fungal fatty acid oxygenases with similarity to ppoA, ppoB and ppoC. Amino acid sequences of the predicted proteins were aligned with ClustalW and the tree was created by TreeView. The scale bar represents 0-1 amino acid substitutions per site. The predicted different oxylipin biosynthetic groups are indicated. Sequence names are indicated according to the annotation performed by the different databases given in Methods.
Deletion of ppo genes resulted in loss of psiB oxylipins

The ppoB gene, encoding a putative fatty acid oxygenase with peroxidase activity, was identified and disrupted in A. nidulans. Chemical analysis of the ΔppoB mutant demonstrated that PpoB, in addition to the previously characterized PpoC (Tsitsigiannis et al., 2004a), is involved in the production of oleic-acid-derived psiBβ (Table 2). Since the production of psiBβ was not totally eliminated in either ΔppoB or ΔppoC strains, it is possible that both oxygenases can utilize oleic acid as a substrate to produce psiBβ. The observation that inactivation of PpoB leads to a different phenotype compared to a ΔppoC strain suggests that parameters other than merely psiBβ concentration determine the outcome of the Aspergillus sporulation program (Table 4). Likewise, the phenotype of the ΔppoΔppoBΔppoC mutant, which only showed trace levels of psiBβ and psiBβ reflecting a non-producing strain, is not easily explained by the elimination of the psiB oxylipin levels alone. Currently we do not know if other oxylipins may be playing a role in A. nidulans development. It is well established that oxylipin-generating enzymes (dioxygenases, oxygenases) may be playing a role in A. nidulans development. It is well established that oxylipin-generating enzymes (dioxygenases, oxygenases, cyclooxygenases) exhibit activity towards more than one substrate. For example, the fungal dioxygenase Lds can oxygenate oleic, linoleic, α-linolenic and ricinoleic acid (Su & Oliw, 1996). It is likely that the Ppo proteins produce several oxylipins, which could also be affecting A. nidulans differentiation processes. Additionally, other factors, such as total fatty acid content and ratio of saturated to unsaturated fatty acids, differ in the Δppo mutant strains (Table 3). These differences may also play an important role in developmental defects of Δppo strains. Previous studies showed that both A. nidulans stearate and oleate desaturase mutants, which also alter the total percentage of fatty acids and saturated to unsaturated fatty acid ratios, affect sexual and asexual spore production (Calvo et al., 2001; Wilson et al., 2004). Additionally, deletion of ppoC led to a significant increase in the transcription of genes involved in fatty acid biosynthesis and a concomitant increase in the total amount of fatty acids in the fungal thallus (Tsitsigiannis et al., 2004a). On the other hand, ΔppoA lowered the transcriptional level of the lipogenic genes, indicating that PpoC and PpoA product(s) regulate signalling cascades that couple meiospore and mitosporogenesis to a host of other developmental programs in A. nidulans, including fatty acid anabolism.

Transcriptional loops are associated with ppo deletions and changes in sexual to asexual spore ratios

Recent physiological and biochemical characterization of PpoA and PpoC mutants revealed a role for oxylipins in maintaining meiospore/mitosporogenesis in A. nidulans (Table 4) (Tsitsigiannis et al., 2004a, b). The characterization of PpoB demonstrates that this protein and/or its products contribute to this process. Here, we discovered that in contrast to ΔppoC, ΔppoB produced significantly higher numbers of conidia but significantly fewer ascospores than the wild-type under both dark and light conditions (Fig. 1). However, as mentioned above, the sporulation phenotypes of the different ppo mutants cannot be explained by psiB oxylipin levels alone as ΔppoB and ΔppoC presented similar psiB profiles (Tables 2, 4 and Tsitsigiannis et al., 2004a). In addition to the changes in fatty acid composition in these strains, our data also indicated that transcriptional alterations in ppo and brlA expression in these mutants are likely to play a significant role in sporulation events.

Deletion of ppoB had profound effects on the transcription of ppoA and ppoC where ppoA was repressed and ppoC upregulated (Fig. 5). As loss of ppoA (Tsitsigiannis et al., 2004b) and presumably overexpression of ppoC (Tsitsigiannis et al., 2004a) results in strains with increased levels of conidia and reduced levels of ascospores, the ΔppoB phenotype could in part be attributed to ppoA and ppoC regulation. This may explain some of the differences in the ΔppoB and ΔppoC phenotypes as loss of ppoC – in contrast to ΔppoB – increased ppoA expression (Tsitsigiannis et al., 2004a). The dependence on each other for normal expression may reflect the existence of direct or indirect regulatory feedback loops among these genes and/or their products that influence meiospore and mitosporogenesis.

Deletion of ppoB clearly increased brlA expression, but had a lesser effect on nsdD expression. brlA encodes a nucleic acid-binding protein with two C2H2 zinc finger motifs whose activity leads to conidiophore formation with terminal differentiation of conidia (Adams et al., 1998; Prade & Timberlake, 1993). Sexual development in A. nidulans requires the GATA-type transcription factor NsdD, necessary for cleistothecia and Hüllle cell production.

Table 4. Comparison of asexual and sexual sporulation and psi factor composition in A. nidulans oxylipin mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Asexual spores</th>
<th>Sexual spores</th>
<th>psiBβ*</th>
<th>psiBo*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>ΔppoA†</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>Trace</td>
</tr>
<tr>
<td>OE::ppoA‡</td>
<td>+</td>
<td>+ + + +</td>
<td>+++++</td>
<td>16+‡</td>
</tr>
<tr>
<td>ΔppoB</td>
<td>+ + + + +</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ΔppoC§</td>
<td>+</td>
<td>+ + + + +</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>ΔppoAC§</td>
<td>+</td>
<td>+ + + + +</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>ΔppoABC</td>
<td>+</td>
<td>12+‡</td>
<td>Trace</td>
<td></td>
</tr>
</tbody>
</table>

*psiBβ, 8-HOE (8-hydroxy oleic acid); psiBβ, 8-HODE (8-hydroxy linoleic acid).
†Described in Tsitsigiannis et al. (2004b).
‡Described number of ‘+’ symbols.
§Described in Tsitsigiannis et al. (2004a).
Ppos regulate the timing and balance of spore development

A striking aspect of the developmental schedule in the homothallic filamentous fungus *A. nidulans* is that the two modes of sporulation are separated in time with asexual preceding sexual development (Adams et al., 1998; Champe et al., 1981, 1994). Generation of the triple *ppo* mutant shifted the timing of mitotic to meiotic development, resulting in sexual development taking precedent over asexual development (Figs 2 and 3). Based on these results, we speculate that oxylipins act as signals that determine the timing and ratio of asexual to sexual differentiation. Our current hypothesis is that the Ppo enzymes and/or their substrates are precisely spatially and temporally regulated in reproductive tissues of the fungal thallus to alter the metabolic profile of cellular oxylipins, which in turn orchestrates the sexual and asexual sporulation schedule. Two lines of evidence support this theory: first, *ppoA* and *ppoC* transcripts are developmentally regulated in differentiated tissues (Tsitsigiannis et al., 2004a, b); and second, microscopic examination of PpoA illustrated that it was localized to metulae of conidiophores and Hülle cells cushioning developing cleistothecia (Tsitsigiannis et al., 2004b). Other observations that may lend support to this hypothesis are derived from studies in *N. crassa*, where oscillation of the molar percentage of the oxylipin substrates linoleic and linolenic acid coincides with oscillation of the circadian rhythm of conidiation (Nukima et al., 1981; Roeder et al., 1982). Moreover, *N. crassa* oleic acid is the predominant fatty acid found in developing ascii and mature ascospores, whereas linoleic acid is the predominant fatty acid in asexual tissue in this fungus (Goodrich-Tanrikulu et al., 1998), suggesting that the availability of Ppo substrates in different tissues could alter the fate of development.

The fact that oxylipins have been implicated in the switch between vegetative and reproductive growth or dimorphism in oomycetes (chronista resembling fungi in life style), yeasts and filamentous fungi (Herman, 1998; Kock et al., 2003; Noverr et al., 2003) augments the above findings in *A. nidulans*. Chemical inhibitors of oxylipin biosynthetic enzymes prevent maturation of the sexual oospore in the chronist *Lagenidium giganteum* (Kerwin et al., 1986) and promote the conversion from mycelial to yeast form in the dimorphic ascomycetous fungus *Ceratocystis ulmi* (Jensen et al., 1992). Our phylogenetic analyses of putative *ppo* orthologues in different fungal species (Fig. 6) may support the existence of a conserved mechanism of oxylipin regulation of fungal development.

Concluding remarks

With the characterization of Ppo proteins we provide genetic evidence of an endogenous lipid-based communication system balancing meiospore and mitospore production in *A. nidulans*. Orthologues of these proteins are found in filamentous and dimorphic fungi and support a case for conservation of an oxylipin-driven mechanism affecting spore development. Considering the ability of plant oxylipins to mimic the phenotype of psi factor application and *ppo* deletion mutants (Calvo et al., 1999), we believe that oxylipins play a significant role in cross-kingdom signalling in host–microbe interactions.

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