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* (Δ*ppoB*) reduced production of the oleic-acid-derived oxylipin psiB\(^\ddagger\) and increased the ratio of asexual to sexual spore development. Generation of the triple mutant Δ*ppoAΔppoBΔppoC* resulted in a strain deficient in producing oleic- and linoleic-acid-derived 8\(^\ddagger\)-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, columella and aggregating sporangiospores), and in the yeast *Dipodascopsis uninnucleata* with the sexual reproductive phase of the life cycle (e.g. gametangia, asci 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 psi\(\ddagger\) and psi\(\ddagger\), 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\(^\ddagger\)-hydroxy-), psiA (5\(^\ddagger\),8\(^\ddagger\)-dihydroxy-) and psiC (designating a lactone ring at the 5\(^\ddagger\) 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 parasticus* (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 component, psiB (Tsitsigiannis et al., 2004b) and the oleic-acid-derived psiB (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 psiBβ 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 decreased (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 inoculating 30 ml liquid GMM with 1 x 10⁸ 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 microscope 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’-GGCGTTGGCTTGCAATAGGG-3’) and ppoB-F4 (5’-ACTCAACAGCCTTCAAACCT-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 kb SalI *brlA*-specific fragment from pTA111 (Adams et al., 1988), a 1.1 kb *nsdD*-specific PCR product obtained with *nsdD*-9 and *nsdD*-3 (Tsitsigiannis et al., 2004a), a 4 kb *ppoB*-specific PCR product obtained with primers ppoA-E2 and ppoA-R2 (Tsitsigiannis et al., 2004b), a 4.1 kb *ppoC*-specific PCR product obtained with primers ppoC-F16 and ppoC-R18 (Tsitsigiannis et al., 2004a) and a 1.1 kb *veA*-specific PCR product obtained with veA-5’ (5’-TTTTGTGTATCCCATCAGATT-3’) and veA-3’ (5’-GTGAGCAGAAGTAGGG-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 TBLASTN 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 *Gaumannimonospora graminis* that was used as query sequence (Hornsten et al., 1999). Oligonucleotides ppoB-F1 (5’-AGTAGGCGTGCGGAGGTG-3’) and ppoB-R1 (5’-AAGGCAGAGTGGTGGTTTG-3’) were designed based on the obtained contig ANI61C010915, predicting a fragment with high identity to LdS and PpoA. These primers were used to amplify a 1.1 kb fragment by PCR, using *A. nidulans* genomic DNA as template. This PCR product was used as a radioactively labelled probe to screen an *A. nidulans* pLORIST genomic cosmid library (Fungal Genetics Stock Center, Kansas City, KS). Two strongly hybridized overlapping cosmids, pLFM13 and pLDG5, were identified and were further used as templates to sequence the entire ORF of the *ppoB* gene as well as approximately 2500 bp of the 5’ and the 3’ flanking regions in both DNA strands. A 6-6 kb KpnI fragment from the cosmid pLFM13 containing the full length of the *ppoB* gene, including its promoter and termination cassette, was subcloned into pBluescript, generating plasmid pTMK1.4.

The *ppoB* deletion construct pTMK8.5, including the *pyroA* marker
Table 1. A. nidulans strains used in this study

<table>
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<tr>
<th>Strain*</th>
<th>Genotype†</th>
<th>Source‡</th>
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<tr>
<td>FGSC237</td>
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</tr>
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<td>biA1 pyroA4 veA1</td>
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<td>biA1 veA</td>
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<td>This study</td>
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<td>RDIT91.7</td>
<td>ΔppoB::pyroA pyroA4 veA ppoB::trpC</td>
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Prototrophic isogenic strains

<table>
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<th>Strain*</th>
<th>Genotype†</th>
<th>Source‡</th>
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<td>Tsitsigiannis et al. (2004b)</td>
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<tr>
<td>RDIT12.9</td>
<td>metG1 ΔppoA::metG veA</td>
<td>Tsitsigiannis et al. (2004b)</td>
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<tr>
<td>RDIT59.1</td>
<td>pyroA4 ΔppoB::pyroA veA</td>
<td>This study</td>
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<td>RDIT58.12</td>
<td>ΔppoC::trpC veA trpC801</td>
<td>This study</td>
</tr>
<tr>
<td>RDIT54.7</td>
<td>ΔppoC::trpC metG1 ΔppoA::metG veA trpC801</td>
<td>Tsitsigiannis et al. (2004b)</td>
</tr>
<tr>
<td>RDIT62.3</td>
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<td>Tsitsigiannis et al. (2004b)</td>
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*Strains starting with ‘T’ are original transformants and ‘R’ are recombinants after sexual cross.
†veA indicates the wild-type veA allele.
‡FGSC, Fungal Genetics Stock Center, Kansas City, KS, USA.

gene and ppoB flanking sequences, was constructed using the following methodology. First, the modified primer pairs ppoB-3DF1-BamHI (5’-CCCT-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-KpnI (5’-CTCAGGATTCGGTACCGTGTCT-3’) were used to PCR-amplify a 1.2 kb flanking region at the 5’ UTR of the ppoB ORF using cosmid pLFM13 as template. The resulting amplified BamHI–KpnI PCR fragment was subcloned into p14 harbouring the pyroA cassette (Osmani et al., 1999), yielding the vector pTMK7.2. Next, the modified primers ppoB-5DF1-HindIII (5’-GGAGAAGACCTTACCGAGCCCT-3’), which is inside the ppoB ORF and 215 bp upstream of the predicted stop codon of ppoB, and ppoB-5DR1-PstI (5’-GGAGAAGACCTTACCGAGCCCT-3’), which is 33 bp downstream of the predicted stop codon of ppoB, were used to PCR-amplify the 1.2 kb flanking region at the 3’ end and 3’ UTR of the ppoB ORF using cosmid pLFM13 as template. The amplified HindIII–PstI 5’ flanking region was further ligated into HindIII–PstI-digested plasmid pTMK7.2, generating plasmid pTMK8.5. The resulting disruption vector pTMK8.5 was used to transform A. nidulans strain RDIT4.4 (Table 1) to pyrodoxime prototroph, creating the transformant TTMK2.60, where the major part of the ppoB ORF was deleted. Gene replacement and ectopic integration were confirmed by PCR and Southern analysis. The ΔppoB allele was introduced in the wild-type veA background by sexual recombination of TTMK2.60 with RDIT55.37 to give the prototrophic strain RDIT59.1. The triple mutant ΔppoAΔppoBΔppoC was created by a sexual cross between RDIT54.13 and TTMK2.60. RDIT44.4 was derived from the cross between RDIT30.32 and FGSC33, RDIT55.37 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 RTMK22.13.

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 protrophs 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) (99:1, 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 follows: 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 70eV, 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/tcb/afu1), Neurospora crassa (www.broad.mit.edu/annotation/fungi/neurospora), Fusarium graminearum (www.broad.mit.edu/annotation/fungi/fusarium), Fusarium verticilloides (www.tigr.org/db/tcb/fcv2), Fusarium sporotrichioides (www.genome.ou.edu/psoro.html), Magnaporthe grisea (www.broad.mit.edu/annotation/fungi/magnaporthe), Phycomyces blakesleeanus (http://genomic.gi-psf.org/whiter01/whiter01.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 linolate 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⁻⁰⁰ 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 cyclooxygenases (GenBank accession numbers: human PGH2, NP_000954; mouse PGH2, 5COX_A; horse PGH2, O19183) and a tobacco pathogen-induced Δ5-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 linolate diol synthase (lds) gene from the filamentous fungus G. graminis var. graminis (Horstien 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 linoleic 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 linolate diol synthase, the Ssp1 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 Ssp1 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 (ΔppoB) 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 ΔppoB strain (RDT59.1) which was used for further physiological and molecular analyses. Both ΔppoB and the triple ΔppoAΔppoBΔppoC mutant had
Table 2. Psi factor composition of mycelia of A. nidulans oxylinopin 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 oxylinopin 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>
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<td>Wild-type‡</td>
<td>5.87 ± 0.70</td>
<td>2.19 ± 0.87</td>
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<tr>
<td>ΔppoB</td>
<td>2.66 ± 0.08*</td>
<td>1.93 ± 0.32</td>
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<tr>
<td>ΔppoAΔppoBΔppoC</td>
<td>0.11 ± 0.11*</td>
<td>0.35 ± 0.33*</td>
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</tr>
</tbody>
</table>

†psiBβ, 8-HOE (8-hydroxy oleic acid); psiBα, 8-HODE (8-hydroxy linoleic acid).
‡Wild-type values were taken with permission from Tsitsigiannis 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)-octadecanoic 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 oxylinopin 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>
</tr>
</thead>
<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>
</tbody>
</table>

†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 Hüle 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 Hüle 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 triple mutant was able to produce Hüle cells 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 Hüle 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 triple mutant was able to produce Hüle cells and heavy cell production.

**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 Δ*ppoB* and Δ*ppoBΔppoC* was correlated with *brlA*, *nsdD* and/or *veA* expression. As shown in Fig. 4, *brlA* transcripts were upregulated in the Δ*ppoB* strain and downregulated in the triple mutant strain. These transcriptional alterations positively correlated with the relative increase (Δ*ppoB*) and decrease (Δ*ppoAΔppoBΔppoC*) in conidial production. Expression analysis of the *nsdD* gene showed that the transcript was slightly upregulated in the ascospore-overproducing strain Δ*ppoAΔppoBΔppoC* and slightly downregulated in the Δ*ppoB* mutant. Interestingly, transcriptional analysis of the *veA* gene demonstrated that is significantly upregulated in Δ*ppoAΔppoBΔppoC* at 48 h (Fig. 4), a time point that coincides with the initiation of Hüle 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 Δ*ppoB* 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 Δ*ppoB* 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 Δ*ppoA* or Δ*ppoC* 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.
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/mitosporal 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.

http://mic.sgmjournals.org
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 ∆ppoB 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 ∆ppoB or ∆ppoC 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 ∆ppoC strain suggests that parameters other than merely psiBβ concentration determine the outcome of the Aspergillus sporulation programme (Table 4). Likewise, the phenotype of the ∆ppoA∆ppoB∆ppoC mutant, which only showed trace levels of psiBx 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) that produce fatty acids and saturated to unsaturated fatty acids, differ in the A. nidulans mutant strains (Table 3). These differences might also play an important role in developmental defects of ∆ppo 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 sporulation 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, ∆ppoA lowered the transcriptional level of the lipogenic genes, indicating that PpoC and PpoA product(s) regulate signalling cascades that couple meiospore and mitosporulation to a host of other developmental programmes 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/mitosporulation balance 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 ∆ppoC, ∆ppoB 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 oxygenin levels alone as ∆ppoB and ∆ppoC 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 ∆ppoB phenotype could in part be attributed to ppoA and ppoC regulation. This may explain some of the differences in the ∆ppoB and ∆ppoC phenotypes as loss of ppoC – in contrast to ∆ppoB – 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 mitosporulation developmental programmes.

Deletion of ppoB clearly increased brlA expression, but had a lesser effect on nbsd expression. brlA encodes a nucleic-acid-binding protein with two C2H2 zinc finger motifs whose activity leads to conidiphore 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 Hülle cell production.

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

The number of ‘+’ symbols indicates approximate and not absolute increase or decrease in spore production or psi factor composition.

<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>∆ppoA†</td>
<td>++ +</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>OE::ppoA‡</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>16+‡</td>
</tr>
<tr>
<td>∆ppoB</td>
<td>++++++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>∆ppoC§</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>∆ppoAC¶</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>Trace</td>
</tr>
<tr>
<td>∆ppoABC</td>
<td>+</td>
<td>12+‡</td>
<td>Trace</td>
<td></td>
</tr>
</tbody>
</table>

*psiBβ, 8-HOE (8-hydroxy oleic acid); psiBx, 8-HODE (8-hydroxy linoleic acid).
†Described in Tsitsigiannis et al. (2004b).
‡Indicates number of ‘+’ symbols.
¶Described in Tsitsigiannis et al. (2004a).
(Han et al., 2001) and the function of the velvet (veA) gene (Kim et al., 2002). VeA is known to have a role in activating sexual development and/or inhibiting asexual development, since asexual sporulation in the veA1 mutant is promoted and increased, while sexual development is significantly delayed and reduced (Kim et al., 2002; Champe et al., 1994). Our experiments demonstrated that the sharp increase in conidiation in the ΔppoB strain was accompanied by a pronounced increase of brlA expression and slight decrease of nsdD transcription (Fig. 4). Changes in sporulation ratios in the ppoA and ppoC mutants were also positively correlated with fluctuations in brlA and nsdD expression (Tsitsigiannis et al., 2004a). In contrast, brlA transcripts were downregulated and veA upregulated in the triple mutant (48 h), a strain that produces fewer conidia and more ascospores compared to wild-type (Fig. 4). The upregulation of veA in the ΔppoAΔppoBΔppoC strain might explain the presence of cleistothecia in liquid media for this mutant since a similar observation was also previously reported in a strain that overexpresses the veA gene (Kim et al., 2002). Additionally, previous studies showed that VeA regulates directly or indirectly the transcription of ppoA gene since in veA1 mutants ppoA was expressed at very high levels only during asexual development and deletion of veA caused inhibition of ppoA transcript accumulation (Tsitsigiannis et al., 2004b). Thus, the results shown here in addition to previous studies (Tsitsigiannis et al., 2004a, b) further support a case for BrlA, VeA and NsdD mediation of Ppo regulation of ascospore and conidial development.

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 oxylinps 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 oxylinps, 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 oxylinp 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 ascini 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 oxylinps 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 oxylinp 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 Ceratoceyistus 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 oxylinp 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 mitospor production in A. nidulans. Orthologues of these proteins are found in filamentous and dimorphic fungi and support a case for conservation of an oxylinp-driven mechanism affecting spore development. Considering the ability of plant oxylinps to mimic the phenotype of psi factor application and ppo deletion mutants (Calvo et al., 1999), we believe that oxylinps play a significant role in cross-kingdom signalling in host–microbe interactions.

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