Endogenous Lipogenic Regulators of Spore Balance in *Aspergillus nidulans*†

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The ability of fungi to produce both meiospores and mitospores has provided adaptive advantages in survival and dispersal of these organisms. Here we provide evidence of an endogenous mechanism that balances meiospore and mitospore production in the model filamentous fungus *Aspergillus nidulans*. We have discovered a putative dioxygenase, PpoC, that functions in association with a previously characterized dioxygenase, PpoA, to integrate fatty acid derived oxylipin and spore production. In contrast to PpoA, deletion of *ppoC* significantly increased meiospore production and decreased mitospore development. Examination of the PpoA and PpoC mutants indicate that this ratio control is associated with two apparent feedback loops. The first loop shows *ppoC* and *ppoA* expression is dependent upon, and regulates the expression of, *nsdD* and *brlA*, genes encoding transcription factors required for meiospore or mitospore production, respectively. The second loop suggests Ppo oxylipin products antagonistically signal the generation of Ppo substrates. These data support a case for a fungal “oxylipin signature-profile” indicative of relative sexual and asexual spore differentiation.

The adaptive success of any organism depends in large part on its ability to sense and respond appropriately to environmental stimuli. Development and survival are highly dependent on proper responses to extracellular signals. In both prokaryotes and eukaryotes a number of developmental signals are derived from common lipogenic origins, suggesting the possibility of a conserved cross kingdom cell-to-cell communication network (11, 20, 42, 56). In the prokaryotic kingdom, several species of lipogenic diffusible molecules regulate a variety of responses (bioluminescence, virulence, biofilm formation, etc.) in a density-dependent manner through the quorum-sensing mechanism (50). In mammals, fatty acid-derived oxylipins (e.g., prostaglandins and leukotrienes) regulate inflammation and other homeostatic responses through an autocrine-paracrine sensing system (19). In plants, similarly structured oxylipins regulate the expression of host defense genes against pathogen and pests and play a major role in the formation of phytohormones and tissue development (5, 17, 34). However, remarkably little is known of lipid signaling in the development and survival of the highly successful kingdom, the Fungi.

Fungi are ubiquitous eukaryotes that are estimated to comprise a quarter of the entire biomass on earth and consist of nearly 1.5 million species, with only 5% identified thus far (25). They are the primary degraders of cellulose and lignin and devastating pathogens of plants and animals. Their success is attributed to their multilateral reproductive strategies, which are uniquely represented by the development of specialized reproductive cells, the meiospore and mitospore. These two spores provide the sexual and asexual modes of fungal reproduction that occur in distinct reproductive organs (3, 4). Sexual reproduction is characterized by the fusion of two nuclei, followed by meiosis and the production of meiospores, and results in a high incidence of genetic recombination and the generation of new genotypes upon which natural selection acts to adapt readily to a multitude of environmental conditions. In many fungi, sexual reproduction usually occurs only once a year and lends adaptive benefits, such as dormancy (overwintering) and drought resistance, to the organism (3, 46, 53). In general, asexual or somatic reproduction is repeated several times during the fungal life cycle, contributing to dispersal by the production of large number of individual mitospores (3, 46, 53). Numerous species in all fungal phyla are able to reproduce both sexually and asexually, and phylogenetic studies indicate this to be the ancestral condition of most taxa (37, 53).

The genus *Aspergillus* comprises a diverse group of species with many members capable of producing only mitospores, a few that produce only meiospores, and several that can produce both spores (49). The homothallic genetic model *A. nidulans* is a classic example of the latter, producing both meiospores (e.g., ascospores) and mitospores (e.g., conidia) (Fig. 1). Studies examining both modes of reproduction in *A. nidulans* describe multiplex tissue development regulated by myriad nutritional and environmental factors, including components of an intrinsic signal transduction pathway that balance vegetative growth with spore development and control the onset of ascosporogenesis and conidiation (2, 6, 11). Ascospore formation in *A. nidulans* requires the GATA-type transcription factor NsdD, necessary for cleistothecia (sexual fruiting bodies bearing the ascospores) and Hülle cell production (21). Conidia formation in *A. nidulans* requires the func-
tion of BrlA, a zinc finger transcription factor essential for conidiophore development (45). Deletion of either gene blocks formation of the respective meiotic or mitotic fruiting body, resulting in a strict asexual morph (ΔnsdD) or a strict sexual morph (ΔbrlA).

In contrast to NsdD and BrlA, which are solely involved in the regulation of the sexual or the asexual cycle, respectively, physiological studies of Champe and el-Zayat (12) led to the identification of secreted lipogenic signal molecules, collectively named “psi factor” (for precocious sexual inducer), that govern the timing and balance of meiotic to mitotic spore development. Biochemical analysis showed that A. nidulans psi factor is an endogenous mixture of hormone-like oxylipins composed of hydroxylated oleic (18:1), linoleic (18:2), and linolenic (18:3) acid molecules termed psiB, psiA, and psiY, respectively (9, 39). The position and number of hydroxylations of the fatty acid backbone further identifies the psi compounds as psiB, psiC, and psia (40). Feeding studies carried out for linoleic acid-derived psiB molecules reported that psiB and psiC stimulated ascospore and inhibited conidial development, whereas psiA had the opposite effect (12, 13). Studies were not performed for purified oleic or linolenic derivatives. A potential role for oxylipins in directing the meiospore-mitospore balance emerged from studies by Tsitsigiannis et al. (55), which identified an A. nidulans dioxygenase (enzymes that catalyze oxygenation of unsaturated fatty acids), PpoA, required for biosynthesis of the linoleic psi factor component, psiB. PpoA localizes in lipid bodies of conidiophores, Hülle cells, and cleistothecia (Fig. 1). Deletion of ppoA significantly reduced the level of psiB and increased the ratio of conidia to ascospores fourfold. In contrast, forced expression of ppoA resulted in elevated levels of psiB and decreased the ratio of conidia to ascospores sixfold. These results correlated with previous studies from Champe’s research group (12, 13).

Here we describe the characterization of another A. nidulans putative fatty acid dioxygenase, PpoC, responsible for formation of the oleic acid-derived psi factor component, psiB. PpoC and PpoA exhibit distinct antagonistic regulation of meiospore and mitosporic development. In contrast to the ΔppoA mutant, deletion of ppoC significantly increased ascospore production and decreased conidial development. ΔppoA and ppoA regulation of spore development appeared to be mediated by brlA and nsdD. Biochemical and transcriptional examination of the PpoA and PpoC mutants also indicated that their products may serve as antagonistic molecular signals of lipogenic genes through regulatory feedback loops in the cellular machinery of the fungus. We hypothesize the existence of a fungal “oxylipin signature-profile” that plays a role in transitions in the human pathogen Candida albicans (43), and recently a bacterial virulence factor structurally similar to farnesolic acid was shown to inhibit the dimorphic transition in C. albicans (56).
Fungal strains, growth conditions, and genetic manipulations. The A. nidulans strains used in the present study (Table 1) were grown on glucose minimal medium (GMM) (9) 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 (44, 58). Developmental cultures were grown on GMM, and asexual and sexual induction was performed as previously described (21, 55). The cultures of RNA shown in Table 1 were grown on solid GMM under dark or white light conditions (prior to harvesting). Radial growth tests were performed in triplicate (dormancy) to mitospore (dispersal) development.

TABLE 1. A. nidulans strains used in this study

| Fungal strain* | Genotype | Source or reference |
|----------------|----------|---------------------|
| RDIT12.3       | biA argB2 metG1 ΔopoA::metG veA | 55 |
| RDIT44.10      | pabaA1 biA1 pyroA4 metG1 veA1 trpC801 | 55 |
| TTMK1.97       | argB2 metG1 ΔopoC::trpC veA1 trpC801 | This study |
| RDIT30.92      | argB2 metG1 veA1 trpC801 | This study |
| RTMK22.13      | pabaA1 biA1 pyroA4 metG1 ΔopoA::metG veA trpC801 | This study |
| RDIT38.21      | argB2 Δopo::trpC pyroA4 metG1 veA trpC801 | This study |
| RDIT35.77      | pyroA4 veA veA trpC801 | This study |
| TDIT11.12      | argB2 Δopo::trpC ppoC::pyroA methG1 veA trpC801 | This study |
| RDIT58.3       | Δopo::trpC pyroA4 veA trpC801 | This study |
| TU85           | pabaA1 biA1 argB2 pyroA4 ΔbrlA::argB veA | L. Yager |
| RDIT2.1        | metG1 veA | This study |
| RDIT86.7       | argB2 ΔbrlA::argB veA | This study |
| KHHS2          | pabaA1 ya2 ΔargB::trpC ΔnsdD::argB trpC801 | 21 |
| KHH62          | pabaA1 ya2 ΔargB::trpC nikA(p)::nsdD trpC801 | 21 |

Prototrophic isogenic strains

| Fungal strain | Genotype | Source or reference |
|---------------|----------|---------------------|
| RDIT9.32      | veA      | 55 |
| RDIT12.9      | metG1 ΔopoA::metG veA | 55 |
| RDIT38.12     | ΔopoC::trpC veA trpC801 | This study |
| RDIT55.7      | Δopo::trpC metG1 ΔapoA::metG veA1 trpC801 | This study |
| RDIT92.6      | ΔapoC::trpC ppoA::pyroA veA trpC801 | This study |
| RDIT81.10     | metG1 ΔapoA::metG veA gpdA(p)::ppoA::trpC | This study |
| RRAW5.2       | argB2 ΔodeA::argB veA | 55 |
| RDIT87.8      | niiA(p)::nsdD veA | This study |
| RDIT88.13     | ΔnsdD::argB veA | This study |

* Strains starting with a “T” are original transformants, and strains beginning with an “R” are recombinants after a sexual cross. Some of the strains are not described in the text but were used for sexual crosses to create the final prototrophic strains.

Materials and Methods

Nucleic acid manipulations. Construction, maintenance, and isolation of recombinant plasmids were performed by using standard techniques (48). Fungal chromosomal DNA was extracted from hyphalized mycelia by using previously described techniques (36). Total RNA was extracted from hyphalized mycelia by using TRIzol reagent (Invitrogen Co.) according to the manufacturer’s recommendations. Approximately 20 μg of total RNA were used for Northern analysis with a 1.2% agarose–1.5% formaldehyde gel transferred to Hybond-XL membrane (Amersham Pharmacia Biotech). The PCR product obtained with primers ppoC-F16 (5'-TTTGTTTTTCTCCGCGGCTC-3') and ppoC-R18 (5'-CA TTAGATACGAAACCAAAGGA-3') with the cosmids PLCJ14 as a template was used as a ppoC DNA probe for Southern and Northern hybridizations.

Molecular cloning, disruption, and cDNA isolation of the A. nidulans ppoC gene. The ppoC gene was identified by tblastn search of the CEREON Genomics A. nidulans database (Monsanto Microbial Sequence Database), based on the amino acid sequence of lipocult die synthase (Lds) cloned from Geotrichum myceae graminis (28) that was used as the query sequence. Oligonucleotides ppoF-1 (5'-ACTACAACCCCCGCAACCTG-3') and ppoC-R1 (5'-GATTTCC TAGTGGCGTGTAGG-3') were designed based on the obtained contig AN616958 predicting a fragment with high identity to Lds and PpoA. These primers were used to amplify a 1.2-kb fragment by PCR, with A. nidulans genomic DNA as a template. This PCR product was used as a probe to screen the A. nidulans pLRIST genomic cosmid library (Fungal Genetics Stock Center, Kansas City, Kans.). Two strongly hybridized overlapping cosmids, pLDFO8 and PLCJ14, were identified and were further used as templates to sequence the entire open reading frame (ORF) of the ppoC gene, as well as ~2.000 bp of the 5’- and the 3’-untranslated flanking regions in both DNA strands. A 9.1-kb SacII-SpeI fragment from the cosmid PLCJ14 containing the ppoC gene was modulating fatty acid biosynthesis and provides a fitness mechanism (46) to the organism by temporally balancing meiospore (dormancy) to mitospore (dispersal) development.
subcloned into pBluescript, generating the plasmid pTMK2.2. The ppoC gene has been assigned accession no. AY613780 in the GenBank database.

The XZAP 24-h developmental cDNA library from A. nidulans (Fungal Genetix Stock Center) was used to isolate the ppoC cDNA according to the supplier's protocols. 5′ and 3′ ends were further confirmed by using RACE (rapid amplification of cDNA ends) technology and the Gene Racer kit (Invitrogen Co.). PCR polymerase (Invitrogen) was used to amplify the corresponding fragments. Sequencing analysis of the resulting clones was performed to determine the positions of the introns and the amino acid sequence of the ppoC gene.

The ppoC deletion construct ppoC::DF58.12 was created by a sexual cross between TTMK1.97 and RTMK22.13 strain RDIT9.32 (55) strain was used as the wild type.

RESULTS

PpoC encodes a putative fatty acid dioxygenase. Disruption of A. nidulans ppoA led to a strain defective in producing the linoleic acid-derived oxylipin psibE (55). To further characterize genes involved in psi factor biosynthesis, we compared the A. nidulans publicly available genome database (Cereon Genomics; Monsanto) with the oxylipin-producing linolate diol synthesis (lds) gene (a ferric hemeprotein with fatty acid dioxygenase and hydroperoxide isomerase activities) from the filamentous fungus G. graminis var. graminis (28). A DNA fragment likely to encode a fatty acid dioxygenase was identified and used to characterize the gene, termed ppoC (for psi-producing oxylipin; GenBank accession number AY613780). Genomic and cDNA analysis showed that PpoC encodes an 1,117-amino-acid polypeptide containing 11 introns (Fig. 2).

Fatty acid analysis. The strains were grown on 15 ml of liquid GMM in petri dishes under stationary conditions at 37°C under a dark regime. Mycelial mats were collected after 72 h, lyophilized, weighted, and homogenized mechanically in 20°C min−1 for 300 min. The ionization current was 100 nA, and the ion source was 280°C. The electron energy was 70 eV, the ionization current was 100 μA, and the scan speed was 0.6 s per decade. Scans were recorded in a range from 35 to 600 atomic mass units (amu).

PpoC also contains a putative hydrophobic subdomain known as a “proline knot” that is characteristic for targeting plant proteins to lipid bodies (29, 55). Taken together, these data suggest that PpoC, three cAMP-dependent protein kinase phosphorylation sites, and several protein kinase C phosphorylation sites. The PpoC amino acid sequence, like those of PpoA and Lds, shared high similarity with the Magnaporthe grisea linolate diol synthase (13) and the Ssp1 protein from Ustilago maydis (29), as well as with various predicted proteins from existing filamentous fungal databases. PpoC orthologs are absent in the yeasts Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Candida albicans but present in the human dimorphic pathogen Histoplasma capsulatum.

Analysis of the PpoC protein by using the PFAM database (http://pfam.wustledu) indicated that the PpoC residues 181 to 650 have domains similar to animal heme peroxidases and residues 765 to 1080 have domains similar to cytochrome P450 oxygenase (Fig. 2). The PpoC peptide contains six possible N-glycosylation sites that might play a role in the maturation of PpoC, three cAMP-dependent protein kinase phosphorylation sites, and several protein kinase C phosphorylation sites. The PpoC amino acid sequence, like those of PpoA and Lds, shared high similarity with various mammalian cyclooxygenases or prostaglandin synthases, ranging from 25 to 26% identity and 38 to 42% similarity. PpoC also contains a putative hydrophobic subdomain known as a “proline knot” that is characteristic for targeting plant proteins to lipid bodies (Fig. 2). PpoA and Ssp1 contain the proline knot motif and are localized to lipid bodies (29, 55). Taken together, these data suggest that PpoC is a paralog of PpoA and G. graminis Lds.

Creation of a ΔppoC strain. A ppoC deletion mutant was generated by replacing the wild-type copy of ppoC with the trpC gene. Random screening of 100 transformants by PCR and Southern analysis revealed three transformants with identical phenotypes showing the DNA size fragments expected for...
a trpC replacement of ppoC (data not shown). One transformant was selected and crossed to produce a prototrophic ΔppoC strain, which was used for further physiological and molecular analyses. The ΔppoA ΔppoC double mutant was also obtained by a sexual cross. Complementation of the ΔppoC strain with a functional copy of ppoC restored the wild-type phenotype, thus confirming that the effects on sexual and asexual sporulation described below were solely due to the deletion of ppoC (data not shown).

**Oxylipin and total fatty acid composition are altered in the ΔppoC strain.** The role of PpoC as a putative fatty acid dioxygenase was explored by analyzing both oxylipin and fatty acid composition of the ΔppoC mutants. Assessment of the two most abundant psi factor components, the oleic acid-derived psiB [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] revealed that deletion of the ppoC allele resulted in almost complete elimination of psiBβ molecules (Table 2). Hydroxylation of the fatty acid backbone designates the psi compounds as psiB [8′ hydroxy-], psiC [5′,8′ dihydroxy-], and psiA [the lactone ring of psiC at 5′ position]) (40). Previous studies showed that deletion of ppoA resulted in a strain deficient in producing psiBα. The double mutant was deficient in the production of both oleic and linoleic acid-derived psiB factors (Table 2). The presence of linoleic or oleic acid-derived psiA or psiC was not detected in any samples in accordance with previous studies (9, 55; data not shown). These data demonstrated that PpoC is involved in the production of psiB oxylipins and suggested that oleic acid is a preferable substrate for PpoC.

Fatty acid composition was analyzed from mycelia grown under dark conditions at 37°C at the same conditions that psi analysis was carried out. Table 3 shows the fatty acid profile of the wild-type and ΔppoA, ΔppoC, and ΔppoA ΔppoC mutant strains. In each strain palmitic, stearic, oleic, and linoleic acids are the most prevalent fatty acids. The ΔppoA strain showed no statistical differences in the amount of the individual fatty acids compared to the wild type. However, ΔppoC showed an increase in palmitic acid and a decrease in stearic acid compared to the wild type. The same pattern was maintained in the double mutant. Deletion of ppoC also led to a twofold increase in the total fatty acid composition per gram of mycelium, whereas ΔppoA strain showed a small but significant decrease in total fatty acid composition. The double mutant did not show a statistically significant alteration in the amount of total fatty acids compared to the wild type.

**PpoA and PpoC antagonistically regulate meiospore and mitosporic development.** Detailed physiological studies of the effect of linoleic acid derived oxylipins on *A. nidulans* development suggested that some components decreased the conidia/ascospore ratio, whereas others had the opposite effect.
In particular, psiB\textsuperscript{a} was implicated in increasing ascospore numbers (12). This finding was genetically supported by examination of two ppoA mutants. Deletion of ppoA resulted in a strain devoid of psiB, whereas the overexpression of ppoA strain (OE::ppoA) overproduced psiB and ascospores (55). Although oleic acid-derived psi factor components were chemically characterized in A. nidulans, bioassays with purified molecules have not been described (9, 39, 40). Here we found that, as with the \( \Delta \)pp oA strain, neither the \( \Delta \)ppoC mutant nor the \( \Delta \)pp oD \( \Delta \)ppoC double mutant had any obvious effects upon the conidia germination (data not shown), growth pattern (Fig. 4A), or morphology of vegetative hyphae (data not shown). However, the kinetics of mitospore and meiospore development was oppositely regulated in \( \Delta \)ppoC and \( \Delta \)pp oA \( \Delta \)ppoC mutants compared to the \( \Delta \)pp oA strain.

Conidia and ascospore production was assessed on GMM under light and dark conditions at 37°C (Fig. 3). Spore production was measured 4 and 6 days after inoculation. In contrast to the \( \Delta \)pp oA phenotype, the \( \Delta \)pp oC and \( \Delta \)pp oD \( \Delta \)ppoC mutants produced significantly fewer conidia but significantly more ascospores than the wild-type strain under both dark and light conditions (\( P < 0.001 \)) (Fig. 3 and 4). These results were maintained over a time period of 10 days (data not shown). Overall, the ratio of conidia to ascospores decreased \( \approx \)3-fold in the \( \Delta \)pp oC mutant and 15-fold in the \( \Delta \)pp oA \( \Delta \)ppoC mutant after 6 days of cultivation in the dark. In contrast, \( \Delta \)pp oA led to a fourfold increase in the conidium/ascospore ratio (55).

Not only were conidia/ascospore ratios decreased in the \( \Delta \)pp oC and \( \Delta \)pp oA \( \Delta \)ppoC strains but development proceeded in an aberrant fashion. Precocious development of Hülle cells, cleistothecia, and ascospores (Fig. 3 and 4) was visibly apparent in these strains, whereas conidiophore development was delayed 4 to 5 h for the \( \Delta \)pp oC and 8 to 10 h for the \( \Delta \)pp oA \( \Delta \)pp oC mutant (Fig. 4A). In radial growth experiments \( \Delta \)pp oC and \( \Delta \)pp oA \( \Delta \)pp oC strains showed approximately 1- and 2-mm retardation of the mature conidiophore zone, respectively. In addition, the \( \Delta \)pp oC and \( \Delta \)pp oA \( \Delta \)pp oC mutants were able to produce Hülle cells and cleistothecia in liquid shake cultures in GMM after 24 h in contrast to \( \Delta \)pp oA mutant and wild-type strains, which are unable to form these structures under these conditions (Fig. 5).

**Changes in the meiotic/mitotic spore ratio are correlated with \( \text{brlA} \) and \( \text{nsdD} \) expression.** Transcriptional regulators specific for each spore stage have been described for A. nidulans. BrlA is a zinc finger transcription factor essential for conidiophore development (45), and NsdD is a GATA-type transcription factor required for cleistothecia development (21). Loss of either locus generates strains unable to produce either conidia (\( \Delta \text{brlA} \)) or ascospores (\( \Delta \text{nsdD} \)), although the alternative spore type is produced normally in these mutants. We were interested in determining whether the changes we observe in ascospore and conidia production in the ppo mutants would be reflected in \( \text{brlA} \) and/or \( \text{nsdD} \) expression.

Mutations in \( \text{brlA} \) result in the “bristle” phenotype, characterized by the fact that conidiophores lack their normal components—vesicles, metulae, phialides, and conidia (Fig. 1). The \( \text{brlA} \) locus consists of overlapping transcription units, designated \( \alpha \) and \( \beta \), with \( \alpha \) transcription initiating within \( \beta \) intronic sequences. As shown in Fig. 6A, the accumulation of \( \text{brlA} \) transcripts showed a temporal delay by \( \approx \)12 to 24 h in the \( \Delta \)pp oC and \( \Delta \)pp oA \( \Delta \)ppoC mutants. In addition, the two different \( \text{brlA} \) transcripts were aberrantly regulated in the \( \Delta \)pp oC mutant, where there is a more pronounced expression of \( \text{brlA} \alpha \) than \( \text{brlA} \beta \) at 48 h. In contrast, an increase of the \( \text{brlA} \) transcripts was observed in \( \Delta \)pp oA at 24 h. These transcriptional changes correlated positively with the relative decrease (\( \Delta \)pp oC and \( \Delta \)pp oA \( \Delta \)ppoC) and increase (\( \Delta \)ppoA) in conidial production.

Deletion of \( \text{nsdD} \) prevents Hülle cell, cleistothecia, and subsequently ascospore formation, whereas overexpression of \( \text{nsdD} \) leads to an increase in sexual development (21). Expression analysis of the \( \text{nsdD} \) gene demonstrated that is upregulated in the ascospore-overproducing strains \( \Delta \)pp oC and \( \Delta \)pp oA \( \Delta \)ppoC especially at 72 h (Fig. 6B), a time point that coincides with the initiation of sexual development in stationary liquid cultures. Loss of \( \text{pp oA} \) showed a slight decrease in \( \text{nsdD} \) expression at 72 h (Fig. 6B).

To further examine possible regulatory interactions between \( \text{ppoA} \) and \( \text{pp oC} \), expression studies were carried out in \( \text{brlA} \) and \( \text{nsdD} \) strains and in strains overexpressing \( \text{nsdD} \) grown in stationary liquid GMM. \( \text{pp oC} \) expression was repressed in \( \text{brlA} \), \( \text{nsdD} \), and OE::\( \text{nsdD} \) strains, whereas \( \text{pp oA} \) expression was not significantly affected in the \( \Delta \text{brlA} \) background at the examined time points but was induced in the \( \text{nsdD} \) background and repressed at 24 h in the overexpression \( \text{nsdD} \) background (Fig. 6C). These results indicated that there is a reciprocal regulation between the \( \text{ppo} \) genes/gene products and these developmental transcription factors.

**Interactive regulatory loops connect \( \text{pp oA} \) and \( \text{pp oC} \) expression.** We considered that the balance of ascospore and conidia production by PpoA and PpoC could implicate a regulatory relationship between these two factors and/or their products. Experiments to test this hypothesis were carried out at the transcriptional level. Previous data demonstrated that \( \text{pp oA} \) expression is correlated with the initiation of asexual and sex-
ual fruiting body formation in *A. nidulans* (55). *ppoC* is not expressed under vegetative conditions (liquid cultures; Fig. 7A, time points 0 and 2), but mRNA studies performed throughout the asexual and sexual life cycle of *A. nidulans* showed that *ppoC* is expressed after induction over a longer time and at significantly higher levels than *ppoA* (Fig. 7A and B). The OE::*ppoA* strain, which accumulates high levels of psiBβ (55), led to a significant suppression of *ppoC* transcript (Fig. 7C1). In addition, mRNA analysis showed that *ppoC* was upregulated and that *ppoA* was significantly downregulated in the ΔodeA mutant (OdeA is a Δ12-oleic acid desaturase converting oleic acid to the polyunsaturated linoleic acid) (Fig. 7C2), a strain that accumulates high levels of psiBβ and no psiBα (9).

**Antagonistic regulation of fatty acid anabolic genes by *ppoA* and *ppoC*.** The fatty acid composition of Δ*ppo* mutants demonstrated that the total percentage of the fatty acids was decreased in the Δ*ppoA* strain and increased in the Δ*ppoC* strain (Table 3). To see whether the biochemical difference was reflected at the transcript level, we examined the expression of several fatty acid biosynthetic genes in the mutant strains grown under the same conditions that fatty acid extraction was performed. Fatty acid synthase α-subunit (*fasA*) encodes the central enzyme in de novo lipogenesis (8), catalyzing the condensation of acetyl coenzyme A (acyt-CoA) and malonyl-CoA into long-chain fatty acids. *fasA* expression was significantly upregulated in the Δ*ppoC* mutant and downregulated in

FIG. 3. Δ*ppoC* and Δ*ppoA* Δ*ppoC* have decreased conidia and increased ascospore production compared to wild-type under both dark and light conditions (*P* < 0.001). Cultures of *A. nidulans* wild-type, Δ*ppoA*, Δ*ppoC*, and Δ*ppoA* Δ*ppoC* were grown at 37°C under dark and light conditions in GMM. Conidium production of 4- and 6-day-old cultures grown in the dark (A) or in light (B) and ascospore production of 4- and 6-day-old cultures grown in the dark (C) or in light (D). Values of ascospores in the dark (4 days) for wild-type, Δ*ppoA* mutant, and Δ*ppoC* mutant strains were low and cannot be represented in the graph (wild type, 390 ± 158; Δ*ppoA*, 120 ± 69; Δ*ppoC*, 330 ± 102). Values are the mean of four replicates, and error bars represent standard errors. Columns with asterisks represent values for the same day that differ significantly from the wild type (*P* < 0.001).
the ΔppoA strain (Fig. 8). This correlated with the total fatty
acid content shown in Table 3. Similar expression patterns
were obtained for the desaturase genes sdeA and sdeB (both
Δ9-stearic acid desaturases converting stearic acid to the
monounsaturated oleic acid) (57), odeA (9), and the oxylipin
biosynthetic gene PpoA (55) (Fig. 8). In contrast, gene expres-
sion was closer to that of wild-type for the ΔppoA ΔppoC
mutant, which did not differ in total fatty acid percentage from
the wild type. It appeared that only the anabolic pathways were
regulated by PpoA and PpoC since expression of foxA, encoding
the catabolic D-bifunctional enzyme (enoyl-CoA hydratase and
hydroxyacyl-CoA dehydrogenase) required for β-oxidation
(38) was not altered in these mutants (Fig. 8).

These results were reminiscent of the feedback regulation of
fatty acid biosynthesis already described in A. nidulans sdeA
and odeA mutants (9, 57) and other eukaryotes, including yeast
(54) and mammals (16). This coordination of lipid homeostasis
is governed by end product feedback regulation of transcrip-
tion. In animals this occurs through the proteolytic release of
transcriptionally active sterol regulatory element binding pro-
teins (SREBPs) from intracellular membranes (47). Mammal-
lian genomes include two SREBP genes (basic helix-loop-helix
leucine zipper transcription factors) that possess considerable
selectivity difference in their target genes and bind to the sterol
regulatory element (SRE) DNA motif in the promoters of
lipogenic genes (46). Polyunsaturated fatty acids, including
oxylipins such as prostaglandins and leukotrienes, appear to
coordinately inhibit lipogenic gene transcription by rapidly re-
ducing the nuclear content of SREBP-1 proteins. Search of the
A. nidulans databases (Whitehead) led to the discovery of two
putative lipogenic transcription factors in Δppo mutants leading to the conclusion that SREBP-1 and
SREBP-2 are significantly upregulated in the ΔppoC strain and
downregulated in the ΔppoA and ΔppoA ΔppoC mutants, thus

FIG. 4. ppoA and ppoC genes are essential for balancing conidiophore/cleistothecia formation. Cultures of A. nidulans wild-type (B1, C1, and
D1), ΔppoA (B2, C2, and D2), ΔppoC (B3, C3, and D3) and ΔppoA ΔppoC (B4, C4, and D4) were grown at 37°C on solid GMM. (A) Deletion
of ppoC delays conidiophore formation. Five-day-old cultures of point-inoculated strains (inoculum, 10⁶ conidia) under light conditions. (B to D)
Induction of the sexual sporulation and suppression of the asexual fruiting bodies in ΔppoC and ΔppoA ΔppoC strains. The opposite is observed
in the ΔppoA mutant. Each strain was inoculated with 10⁶ conidia/plate, and cultures were grown for 2 days under dark (B), 8 days under dark (C),
and 8 days under light (D) conditions. Black balls are cleistothecia (“CI” in panel D3), fuzzy balls are cleistothecia initials (“CI In” in B4), and
smaller green spheres are conidiophore heads.
likely mediating the regulation of the anabolic genes involved in the fatty acid metabolism described above (Fig. 8). These results indicated that oxylipins may play a regulatory role in \textit{A. nidulans} fatty acid metabolism as they act in mammals.

**DISCUSSION**

A central issue in fungal biology lies in elucidating the exogenous and endogenous factors required for meiospore and mitospore reproduction. Aside from their pivotal roles in fungal dissemination and survival, fungal spores comprise mainly the primary and secondary infection particles of plant pathogenic fungi (3, 4). Usually, the asexual spore serves as both primary and secondary inoculum of infection, whereas in some ascomycetes the overwintering sexual spore is the source of primary inoculum (3, 4). The experiments presented here show two genes that can influence the process of meiospore and mitospore development relative to each other. In the present study, we characterized \textit{ppoC}, encoding a putative fatty acid dioxygenase in the model organism \textit{A. nidulans}. PpoC is involved in the production of oleic acid-derived psi factor and has an opposing function to the previously characterized \textit{PpoA}. Both enzymes serve as essential signaling regulators of mitotic-meiotic spore balance. To our knowledge, this is the first genetic study identifying a mechanism integrating an antagonistic orchestration of asexual and sexual reproduction.

\textbf{PpoC is involved in the production of oleic acid oxylipins.} Chemical analysis of the \textit{\textDelta ppoA}, \textit{\textDelta ppoC}, and \textit{\textDelta ppoA \textDelta ppoC} mutants demonstrated that PpoC, in contrast to the previously characterized PpoA, is probably involved in the production of psiB\textbeta. The double mutant is crippled in its ability to produce both psiB\textalpha and psiB\textbeta (Table 2). However, we cannot exclude the possibility that PpoA and PpoC are involved in the production of other oxylipin species: either downstream products of psiB\textalpha and psiB\textbeta and/or derivatives of alternative fatty acids. Oxylipin-generating enzymes (dioxygenases, lipoxygenases, cyclooxygenases, etc.) frequently exhibit activity toward more than one substrate. For example, the \textit{G. graminis} dioxygenase Lds can oxygenate oleic, \textalpha-linolenic, and ricinoleic acid, as well as linoleic acid (52). Since the double mutant still produces some psiB molecules, our results also suggest the presence of another enzyme capable of generating these products. This is similar to the situation in plants in which several lipoxygenases are involved in producing the same oxylipins (reference 17 and references therein).

\textbf{Regulatory links between ppoA and ppoC: mechanism for an oxylipin signature.} Champe’s physiological and biochemical characterization of psi factor in the 1980s was among the first studies to uncover a mechanism that shifted the meiospore-mitospore balance in fungi and was certainly the first to implicate the role of oxylipins in this phenomenon (12, 13). The phenotype of the \textit{ppoA} mutant seemingly supports his findings. However, since Champe and coworkers did not assay the effects of purified oleic acid oxylipins on \textit{A. nidulans}, we are unable to directly compare the \textit{\textDelta ppoC} phenotype to any published work. We also note that the application of a purified metabolite differs in many respects to the absence of a gene. Because deletion of \textit{ppoA} and \textit{ppoC} showed pleiotropic effects, including changes in oxylipin make-up, fatty acid composition, and gene expression, we hesitate to attribute a particular role to a single oxylipin species. Rather, we suggest that
oxylipin molecules as a whole are important in generating differentiation processes in *A. nidulans* in a manner similar to that described in plants.

In-depth analyses of plant oxylipin pathways have uncovered complex and tight control of oxylipin production, presumably required for appropriate development in changing environmental milieus (5). The temporal and spatial activity of different oxylipin biosynthetic enzymes appears to be of fundamental importance for normal growth; this is especially true for lipoxygenase isoforms (17). Activities and compartmentalization of the biosynthetic oxylipin enzymes is of paramount importance in determining the oxylipin profiles that will lead to the appropriate developmental pathway. Recent analyses indicate that the phyto-oxylipin pool of a given organelle, tissue, plant, or species confers an “oxylipin signature” to that respective entity (24, 35). It is proposed that the oxylipin signature is predictive of the execution of specific developmental pathways for the organism (5, 17).

Our findings suggest a similar conserved complex control exists in fungi and that a fungal oxylipin signature could be predictive of meiospore and mitospore development in any given fungal isolate. The expression profiles of *ppoA* and *ppoC* suggested that *ppoA* transcripts are important for the initiation of conidiophores and cleistothecia but *ppoC* needs to be at significantly higher transcriptional levels after asexual induction for normal sporulation (Fig. 7A and B). Inhibition of *ppoC* expression in a *ppoA* overexpression strain suggests the possibility of feedback regulation between *ppoA* and *ppoC*, ostensibly via oxylipin production (Fig. 7C and Fig. 8). This latter point is supported from results showing suppression of *ppoA* and induction of *ppoC* in a *ΔodeA* strain (Fig. 7C). Inactivation of *OdeA* (9), a Δ-12 desaturase required for linoleic acid biosynthesis, results in a strain that produces a sixfold increase in the oleic acid-derived psiB but no psiBα. We speculate that fungal oxylipin production is dependent on stimuli that can lead to alterations of the developmental schedule to withstand adverse or favorable environmental conditions.

**BrlA and NsdD: mediators of oxylipin signaling?** The abnormal sporulation patterns of the *Δppo* mutants led us to investigate the expression profiles of the major developmental transcription factors BrlA and NsdD. The proper expression of *brlA* during asexual spore formation in *A. nidulans* is critical for the development of the conidiophores and for the activation of other developmentally specific genes (2). Our experiments in-

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**FIG. 6.** BrlA and NsdD are involved in the regulation of meiospore/mitospore ratio in *A. nidulans*. (A and B) Gene expression analysis of the spore specific transcriptional regulators *brlA* (asexual) (A) and *nsdD* (sexual) (B) in wild-type and *ΔppoA, ΔppoC*, and *ΔppoA ΔppoC* mutant strains. (A) Temporal delay of *brlA* transcripts (α and β) in the conidium-deficient *ΔppoC* and *ΔppoA ΔppoC* mutant strains. (B) *nsdD* gene is upregulated in the ascospore-overproducing *ΔppoC* and *ΔppoA ΔppoC* mutant strains. (C) *ppoA* and *ppoC* expression is altered in *ΔbrlA, ΔnsdD*, and overexpression *nsdD* (OE::*nsdD*) strains. Strains were grown in stationary liquid GMM at 37°C, and mycelia were harvested at the indicated time points. Equal loading of total RNA (20 μg) is depicted by ethidium bromide staining of the rRNA.
dicated that the delay in conidiation in \( \Delta ppoC \) and \( \Delta ppoA \) mutants was at least partially mediated by the delay and alteration in the expression of \( \text{brlA} \) that are individually essential for the formation of morphologically normal conidiophores (22, 23). \( \text{brlA} \) contains two ORFs: a small ORF, \( \text{ORF}_1 \), that is located upstream of the \( \text{brlA} \) initiation codon, and a large ORF that encodes \( \text{BrlA} \). The translation of \( \text{ORF}_1 \) inhibits the translation of \( \text{brlA} \) that in turn is required for \( \text{brlA} \) transcription, leading to the activation of a series of conidiation genes. In our studies, \( \text{brlA} \) was more abundant in \( \Delta ppoC \) mutants (Fig. 6A), indicating a role for PpoC and/or its enzymatic products in regulating the transcriptional ratio of \( \text{brlA} \). Examination of the promoter regions of \( ppoA \) and \( ppoC \) revealed the presence of several putative BrlA response elements (data not shown) (14), further supporting our results that the transcriptional regulation of \( ppoA \) and \( ppoC \) is under the control of BrlA. In contrast to \( brlA \) expression, expression of the sexual stage transcription factor \( \text{nsdD} \) was upregulated in the ascospore overproducing \( \Delta ppoC \) and \( \Delta ppoA \) \( \Delta ppoC \) strains and downregulated in the \( \Delta ppoA \) mutant, a strain that produces fewer ascospores than does the wild type (Fig. 6B). These results suggest that Ppo regulation of ascospore and conidial development is at least partially mediated through the \( \text{nsdD} \) and \( brlA \) transcription factors as summarized in our proposed model in Fig. 9.

Our studies also support a case for reciprocal regulation of \( ppo \) expression by BrlA and NsdD. Figure 6C shows that BrlA

FIG. 7. \( ppoC \) and \( ppoA \) are differentially regulated under asexually (A) and sexually (B) induced cultures. Mycelia of the wild-type strain were synchronized by 18 h of vegetative growth in liquid shaken GMM (time zero) and developmentally induced on solid GMM to obtain asexual (A) and sexual (B) tissue types for RNA isolation at appropriate time intervals under dark or light conditions. Time points represents hours after asexual or sexual induction, respectively. Induction of asexual sporulation was performed under normal aeration conditions. The time point “−2” corresponds to 16 h of vegetative growth culture, 2 h before the transfer to solid medium for asexual induction. For the induction of sexual sporulation 18-h-liquid-grown mycelia were transferred onto solid medium, and the plates were sealed with parafilm for 20 h. After 20 h, the plates were unsealed, and at different time points samples were collected for RNA analysis (time represents hours after induction: 0 to 36 h). The time point “T” corresponds to the sample that was collected at the time of transfer to the solid media and before the initiation of sexual induction. (C) Differential regulation of \( ppoA \) and \( ppoC \) expression as was demonstrated by their transcript analysis in \( \text{OE-ppoA} \) (C1) and \( \text{odeA} \) (C2) strains. Equal loading of total RNA (20 \( \mu g \)) is depicted by ethidium bromide staining of the rRNA. The time points of mycelium harvest are indicated above the lanes.
acts as a positive regulator of ppoC and a negative regulator of ppoA and NsdD acts as a negative regulator for both ppoA and ppoC. Based on these results we speculate that ppoC is regulated by BrlA and NsdD through a feedback mechanism and serves as a positive regulator of asexual and negative regulator of sexual development (Fig. 9). The fact that both BrlA and NsdD act as negative regulators of ppoA and that brlA and nsdD expression was not greatly affected by the ppoA deletion may indicate that ppoA acts downstream or in different pathways at transcriptional or translational levels to regulate the asexual and sexual cycles.

This apparent feedback loop between ppoA-ppoC and nsdD-brlA indicates a mechanism by the organism to maintain tight control of the meiospore/mitospore ratio and eliminate the possibility of a failure in the developmental mechanism. We conjecture that this interaction occurs in the reproductive tissues of the fungus. Our previous study demonstrated that PpoA accumulates predominantly in lipid bodies found in Hülle cells, nascent cleistothecia, and mature conidiophores—the tissues containing the highest concentrations of lipid bodies (55). PpoC, like PpoA, contains a proline knot motif in the central domain of the polypeptide (Fig. 2) that is crucial for targeting and anchoring proteins to lipid bodies (41) and is also likely to localize to these same tissues. Although no studies are available showing BrlA or NsdD localization, it is logical to assume that they would also be present in these tissues, which are dependent on their function.

**Transcriptional regulation of lipid homeostasis.** 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. On the other hand, ΔppoA lowered the transcriptional level of the lipogenic genes. Studies in primary rat hepatocytes and cultured 3T3-L1 adipocytes showed that arachidonic acid-derived oxylipin metabolites (e.g., prostaglandin E₂) suppress the expression of the fatty acid synthase (FAS) through a G-protein-coupled receptor prostanoid signal transduction cascade (30). Thus, in analogy to these studies, we hypothesize that PpoC and PpoA product(s) modulate SREBP expression indirectly, perhaps by instigating autocrine-paracrine antagonistic signaling cascades that couple meiospore and mitospore production to a host of other developmental programs in *A. nidulans*, including fatty acid anabolism.

**Conclusions.** With the characterization of ppoA and ppoC, we provide evidence of an endogenous system balancing meiospore and mitospore production in *A. nidulans*. Orthologs of both of these genes are found in filamentous fungi and, coupled with the numerous studies linking oxylipin production with fungal sporulation (27, 31, 33, 42, 51), support a case for conservation of an oxylipin-driven mechanism controlling,
among other cellular processes, sexual and asexual differentiation. We propose that fungal oxylipins serve as autocrine or paracrine signals generated in response to—and enabling the fungus to respond appropriately to—specific environmental parameters. Since previous studies showed that *Aspergillus* spp. respond to seed oxylipins in a manner similar to that of psiB/H9251 and psiB/H9252 (10), it is reasonable to postulate that host oxylipins can mimic and/or interfere with endogenous fungal oxylipins on a cellular basis, thus affecting the outcome of the host-fungal interaction. Recent evidence suggests that endogenous unsaturated fatty acids regulate morphological transitions and virulence in *C. albicans* (43) and have structural and functional homologs in prokaryotes (56), suggesting that fatty acids or the downstream oxylipins act as signals of cross-kingdom cell-cell communications. It remains to be examined whether microbial oxylipins act as virulence factors in these same interactions. Noverr et al. (42) postulated that microbial oxylipins can modulate disease pathogenesis and host immunity responses. Certainly, a better understanding of the molecular mechanisms that govern fungal oxylipin metabolism could contribute to the design of novel chemicals or other strategies that can reduce the survival and spread of pathogenic fungi.

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