The Lipid Body Protein, PpoA, Coordinates Sexual and Asexual Sporulation
in Aspergillus nidulans*

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**SUMMARY**

The coexistence of sexual and asexual reproductive cycles within the same individual is a striking phenomenon in numerous fungi. In the fungus *Aspergillus nidulans* (teleomorph: *Emericella nidulans*) endogenous oxylipins, called psi factor, serve as hormone-like signals that modulate the timing and balance between sexual and asexual spore development. Here, we report the identification of *A. nidulans ppoA*, encoding a putative fatty acid dioxygenase, involved in the biosynthesis of the linoleic acid derived oxylipin psiBα. PpoA is required for balancing anamorph and teleomorph development. Deletion of *ppoA* significantly reduced the level of psiBα and increased the ratio of asexual to sexual spore numbers four fold. In contrast, forced expression of *ppoA* resulted in elevated levels of psiBα and decreased the ratio of asexual to sexual spore numbers six fold. *ppoA* expression is mediated by two developmental regulators, VeA and the COP9 signalosome, such that *ppoA* transcript levels are correlated with the initiation of asexual and sexual fruiting body formation. PpoA localizes in lipid bodies in these tissues. These data support an important role for oxylipins in integrating mitotic and meiotic spore development.
INTRODUCTION

A unique property of many fungi is their ability to propagate by both sexual and asexual spores. The integration of the teleomorph (meiotic sexual morph) and anamorph (mitotic asexual morph) stages into the fungal life cycle lends great flexibility in dispersal and survival during suboptimal environmental conditions, both very important characteristics for organisms that are essentially sessile in their somatic stage. *Aspergillus nidulans* (teleomorph: *Emericella nidulans*) is a homothallic ascomycete with a defined asexual and sexual cycle that has long served as model system for understanding the genetic regulation of asexual development and secondary metabolism in filamentous fungi (1, 2). The asexual cycle is characterized by the production of haploid conidiophores that bear single-cell asexual spores called conidia. Sexual development commences with the formation of multinucleate globular cells, called Hülle cells that surround the cleistothecium, the ascocarp that contains sexual spores called ascospores.

The program of asexual and sexual sporulation is characterized by many developmental stages including temporal and spatial regulation of gene expression, cell specialization and intercellular communication (1, 2). The *A. nidulans* asexual reproductive cycle can be divided into at least three different stages: (i) a growth phase required for cells to acquire the ability to respond to induction signals (competence phenomenon), (ii) initiation of the developmental pathway and (iii) execution of the developmentally regulated events leading to sporulation. A development-specific array of transcription factors is activated that control the expression of multiple sets of genes required for conidiophore morphogenesis (1). Sexual fruiting body formation is influenced by several environmental and genetic determinants; however, the molecular pathways for this developmental stage are not well dissected (3). Current studies are
focusing on the characterization of mutants defective in sexual reproduction (4). Normal sexual and asexual development in *A. nidulans* requires the function of the velvet (*veA*) gene (5). 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 (5-7). Furthermore, *veA1* mutants do not exhibit light-dependent development of conidia and ascospores in contrast to the wild type where light induces asexual and delays and reduces sexual spore production (7). The *veA1* mutant gene differs from that of the wild type by one nucleotide in the initiation codon resulting in a putative truncated protein by 37 amino acids. *veA* null (Δ*veA*) mutants do not form cleistothecia; by contrast, overexpression of *veA* gene leads to formation of cleistothecia even in liquid culture and to formation of fewer conidial heads than in a wild type on solid media (5).

Butnick *et al.* (8) identified two mutants (*acoB202, acoC193*) in *A. nidulans* that fail to become competent and are blocked in both sexual and asexual sporulation. These mutants overproduce hormone-like fatty acid derived oxylipins collectively termed psi factor (precocious sexual inducer). Psi factor serves as signals that modulate sexual and asexual sporulation by affecting the timing and balance of asexual and sexual spore development (9-11). Biochemical analyses revealed that psi factor is composed primarily of hydroxylated oleic (18:1) and linoleic (18:2) moieties called psiβ and psiα respectively (10-13). The positioning of the hydroxy groups on the fatty acid backbone further designates the psi compounds as psiB (8’ hydroxy-), psiC (5’, 8’ dihydroxy-) and with psiA designated for a lactone ring of psiC at 5’ position (Fig. 1). Studies, only carried out for the linoleic acid derived psiα molecules, showed that psiBα and psiCα stimulated sexual and inhibited asexual spore development whereas psiAα had the opposite effect (9, 10). PsiBα (8-hydroxylinoleic acid or 8-HODE) has also been previously isolated from
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*Gaeumannomyces graminis* (14) and the basidiomycete *Laetisaria arvalis* (15), where it was noted for its biocontrol activity against soil plant pathogens including *Rhizoctonia solani* and *Pythium ultimum* (16). 8-HODE is produced by a well biochemically characterized cytosolic oxygenase, linoleate 8-dioxygenase, in both *G. graminis* and *L. arvalis* (17, 18). However, the role of this enzyme in fungal development is not known.

Our interest in psi factor stems from its role as a signal in *Aspergillus* sexual and asexual sporulation (9-11). To gain more insight into the relationship between morphological development and psi factor/oxylin biosynthesis we have initiated studies to identify and characterize fatty acid dioxygenases in *A. nidulans* responsible for the production of oxylipins. Here we describe PpoA, a putative dioxygenase localized to lipid bodies in asexual and sexual fruiting structures. Deletion of *ppoA* reduced the level of the linoleic acid derived psiBα and increased the ratio of asexual to sexual spore development four fold. On the other hand, forced expression of *ppoA* resulted in elevated levels of psiBα and decreased the ratio of asexual to sexual spore development six fold. Previous studies have shown similar results by adding exogenous pisBα in confluent mycelial cultures (10). Normal expression of *ppoA* required the two developmental regulators, VeA and COP9 signalosome. This is the first study that shows at a genetic level the direct involvement of oxylipins in spore development in *A. nidulans*.

**EXPERIMENTAL PROCEDURES**

**Fungal strains and growth conditions**

*Aspergillus nidulans* strains used in this study are listed in Table I. Strains were grown on glucose minimum medium (GMM) (11) with appropriate supplements for the corresponding
auxotrophies, at 37 °C in continuous dark or in continuous white light. Illumination was carried out in an incubator equipped with General Electric 15-W broad-spectrum fluorescent light bulbs (F15T12CW) placed 50 cm below the plates. Developmental cultures were grown on GMM and asexual and sexual induction was performed as previously described (4). Sexual crosses of \textit{A. nidulans} strains were conducted according to Pontecorvo \textit{et al.} (19). Fungal transformation was carried out as previously described (20) with the modification of embedding the protoplasts in 0.75% top agar rather than spreading them by a glass rod on solid media.

**Nucleic acid manipulations**

Standard methods were used for construction, maintenance, and isolation of recombinant plasmids (21). Fungal chromosomal DNA was isolated and analyzed from lyophilized mycelia using previously described techniques (22). Total RNA was extracted from lyophilized mycelia using Trizol reagent (Invitrogen Co.) according to manufacturer’s recommendations. Oligonucleotides used in this study are listed in Table II. The PCR product obtained with primers ppoA-F2 and ppoA-R2, using pWF116 as template, and the \textit{XbaI} fragment from pDIT1.1, were used as DNA probes for Southern and northern hybridizations. Nucleotide sequences were analyzed and compared using Sequencher (Gene Codes Co. MI, USA.) and ClustalW (http://www.ebi.ac.uk/clustalw/) software programs.

**Plasmid and strain construction**

The amino acid sequence of \textit{Gaeumannomyces graminis} linoleate diol synthase (Lds) was used to identify the \textit{ppoA} gene by tblastn search of the Cereon Genomics \textit{A. nidulans} database (Monsanto Microbial Sequence Database: http://microbial.cereon.com). Oligonucleotides ppoA-F1 and ppoA-R1 were designed based upon a 3558-bp \textit{A. nidulans}
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Genomic sequence (contig ANI61C3259), predicting a fragment with high identity to Lds. These primers were used to amplify an 1100-bp fragment by PCR, using *A. nidulans* genomic DNA as template. This PCR product was used as a probe to screen the *A. nidulans* pLORIST and pWE15 cosmid libraries (Fungal Genetics Stock Center, Kansas City, KS). Two strongly hybridized cosmids, pLEE10 and pWF116, were identified and found to be overlapping by subsequent restriction analysis. A 6300-bp *XbaI* fragment from the cosmid pWF116 harboring the *ppoA* gene was subcloned to pBluescript SK− (Stratagene, CA, USA) generating the plasmid pDIT1.1. Cosmid pWF116 and plasmid pDIT1.1 were used as templates to sequence the entire ORF of the *ppoA* gene as well as ~1500-bp of the 5’ and the 3’ untranslated flanking regions in both DNA strands. The *ppoA* gene has been deposited in the GenBank database under GenBank Accession Number AY502073.

Total RNA was extracted from 24h-liquid shake cultures from strain RDIT9.32 for *ppoA* cDNA. mRNA was purified using the Oligotex mRNA Isolation kit (Qiagen) and cDNA was synthesized by reverse transcriptase-PCR (RT-PCR) using RACE technology and the Gene Racer Kit (Invitrogen Co.) following the supplier’s protocols. *ppoA* primers and Pfu polymerase (Stratagene, CA, USA) were used to amplify the *ppoA* fragments that were subcloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen Co.). Sequencing analysis of the resulting clones was performed to determine the cDNA sequence of the *ppoA*.

The *ppoA* deletion plasmid pDIT3.11 included the *metG* marker gene and *ppoA* flanking sequences without the *ppoA* encoding region, was created as follows: First, the primers ppoA-5DF3 and ppoA-5DR1 were used to PCR amplify a 1100-bp flanking region at the 5’ UTR of the *ppoA* ORF using the cosmid pWF116 as template. ppoA-5DF3 and ppoA-5DR1 were designed to introduce *XbaI* and *XmaI* restriction sites at either side of the PCR fragment respectively. The
amplified *XbaI-Xmal* product was subcloned into pBluescript SK− yielding the vector pDIT2. Next, the primers ppoA-3DF1 and ppoA-3DR1 were used to amplify the flanking region at the 3′UTR of the *ppoA* ORF using the cosmid pWFI16 as template. ppoA-3DF1 and ppoA-3DR1 were designed to introduce *SphI* and *KpnI* restriction sites at either side of the PCR fragment respectively. Finally, the amplified *SphI-KpnI* 3′ flanking region and the 2470-bp *Xmal-SphI* fragment from the pUG11-41, containing the *A. nidulans metG* cassette (23) were ligated at the same time into the *Xmal-KpnI* sites of the pDIT2. The resulting disruption vector pDIT3.11 was used to transform the *A. nidulans* strain pW1 to methionine prototrophy, creating the transformant TDIT2.9 where the entire *ppoA* coding region was deleted. Gene replacement and ectopic integration were confirmed by PCR and Southern analysis. The Δ*ppoA* allele was introduced in a veA background by sexual recombination of TDIT 2.9 with RDIT2.30 to give the prototrophs RDIT12.1 and RDIT12.9.

The vector pDIT19.4 was used to complement the *ppoA* deletion strain RTMK22.28. The plasmid pDIT19.4 was created by inserting a 6300-bp *Xmal-NotI* fragment from the plasmid pDIT1.1 containing the promoter, the coding sequence and the termination cassette of *ppoA*, into pJW532. pJW53 contains an 1800-bp fragment of the 5′ portion of the *A. nidulans* pyridoxine biosynthetic gene (*pyroA*), which can complement the *pyroA4* mutation by single crossing over. The pyridoxine prototroph TDIT8.1 containing the *ppoA::pyroA* allele was isolated. RDIT83.1 and RDIT83.2 are the prototrophic meiotic progeny of the cross between TDIT8.1 and RDIT55.27.

To overexpress the *ppoA* gene, modified primers ppoA-F15-KpnI and ppoA-R18-KpnI were used to PCR amplify the region of the *ppoA* locus from pWFI16. The resulting 4000-bp *KpnI* fragment spanning the region 55-bp upstream of the start codon to the polyadenylation site,
was subcloned into both pRB1 (24) and pDIT8.4 generating the plasmids pDIT10.9 and pDIT11.3 respectively. pRB1 contains a 1750-kb fragment of the 5' portion of the *A. nidulans* trpC gene, which complements the trpC801 mutation, and niiA(p), the nitrite reductase promoter, which is suppressed by ammonium and induced by nitrate. pDIT8.4 was created by replacing the NotI-NcoI fragment containing the niiA promoter from pRB1 with an amplified NotI-NcoI fragment of the *A. nidulans* constitutively expressed glyceraldehydes-3-phosphate dehydrogenase promoter gpdA (25). The gpdA(p) was PCR amplified using the modified primers gpdA-5-NotI and gpdA-3-NcoI from pAN52.3 (25). pDIT10.9 and pDIT11.3 were introduced into RDIT30.92 and RDIT30.12 respectively. Tryptophan prototrophs containing the niiA(p)::ppoA or the gpdA(p)::ppoA construct were isolated based on PCR and Southern analysis and named TDIT4.2 and TDIT5.8 respectively. The niiA(p)::ppoA and the gpdA(p)::ppoA alleles were introduced in a ΔppoA; veA background by sexual recombination of TDIT4.2 with RTMK22.16 and TDIT5.8 with RDIT22.16 to give the prototrophs RDIT75.24 and RDIT81.10. Overexpression of ppoA (OE::ppoA) was also confirmed by gene transcript analysis.

The sgfp-ppoA fusion protein (sgfp = synthetic green fluorescent protein) (26) was constructed in two steps. First, primers NGPPOA-5’GFP and NGPPOA-3’GFP, constructed with KpnI and SphI sites respectively, were used to amplify a 740-bp fragment encoding sGFP from the plasmid pPRgf-T4 (kindly provided from C. Cortez). Second, modified primers NGPPOA-5’ppoA and ppoA-R18 amplified a 4125-bp SphI–KpnI ppoA PCR product from pWFI16. Finally the KpnI-SphI gfp fragment and the SphI-KpnI ppoA fragment were ligated into the KpnI digested pDIT8.4 vector adjacent to the gpdA(p). The generated plasmid, pDIT17.1, had the 1100-bp fragment of the 5' portion of the *A. nidulans* trpC gene next to the gpdA(p)::gfp-ppoA allele. The GFP construct was inserted by transformation as a single ectopic integration into the
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*A. nidulans trpC* locus of the strain RDIT30.23 yielding the transformant TDIT7.3. A sexual cross between TDIT7.3 and RDIT12.15 led to the meiotic prototrophic progeny RDIT71.8. The fusion construct *gpdA(p)::gfp-ppoA* was further confirmed by gene transcript analysis. The plasmid pCV10 carrying the *gpdA(p)::gfp* fusion was introduced into the *A. nidulans* RDIT 55.31 strain yielding the transformant TDIT9.14 that used as control in the GFP experiments.

**Lipid extraction and fatty acid analysis**

Wild type, ΔppoA and ppoA overexpression strains were grown in 25 ml of liquid GMM in Petri dishes under stationary conditions at 37°C in the dark. Mycelial mats were collected after 72h, freeze-dried, weighed and homogenized mechanically using Ultra-Turrax®T25 dispenser (Ika Werke GmbH & Co. KG). Lipids were extracted and converted into fatty acid methyl esters (FAME) derivatives using 2% sulphuric acid in methanol as described elsewhere (27). FAME were converted into corresponding trimethylsilyl ether (OTMSi) derivatives using BSTFA (Supelco, 33154-U) as a silylation agent according to the method reported previously (28). Samples were dissolved in 200 μl of hexane/chloroform (4:1) and stored at –20°C until further laboratory analyses. OTMSi-FAME were analyzed by flame ionization detection-gas chromatography (FID-GC) and GC-EI/MS as described by Ghanevati and Jaworski (29). FAME were analyzed on a Hewlett-Packard 6890 gas chromatograph fitted with a 30-m J&W DB-225 capillary column (Agilent, Palo Alta, CA) and connected to a double flame ionization detector set at 300°C. GC/MS measurements were made with a KRATOS Analytical (Manchester, UK) MS 25 RFA mass spectrometer equipped with a Carlo Erba (Milan, Italy) Model 5160 gas chromatograph. A J&W Scientific (Folsom, Ca., USA) fused-silica capillary GC column (30 mm x 0.32 mm I.D.) with DB-5MS coating (0.25 μm) was used. Nonadecanoic acid (19:0) was used
as an internal standard. Fatty acids were identified by comparison of retention times with a set of authentic fatty acid standards, whereas 8-hydroxyoleic (8-HOE) and 8-hydroxylinoleic (8-HODE) acids were identified by mass spectrometry on the basis of their fragmentation patterns (11, 30).

**Physiological studies**

All strains used for physiological studies were prototrophic. The wild type RDIT9.32 was a prototrophic meiotic progeny of a sexual cross between WIM126 and RDIT1.7. Asexual and sexual spore production studies were performed on plates containing 30 ml of solid 1.5% GMM or YGT media. GMM is a medium known to promote the asexual spore stage and YGT medium has been used in previous research to promote sexual development in *A. nidulans* (9, 10, 31). Five ml of top layer with cool melted 0.7% agar-GMM or YGT that contained 10⁶ conidia of the appropriate strain was added on each plate. Cultures were incubated in continuous dark or light at 37 °C. 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 of sterile water supplemented with 0.01 % Tween 80 to release the spores. Spores were counted using a hemacytometer. The experiments were performed with four replicates. Induction of *niiA* promoter was performed as described previously (24). Spore data and fatty acid analysis results were statistically compared by analysis of variance (ANOVA) and Fisher’s Least Significant Difference (LSD) using the Statistical Analysis System (SAS Institute, Cary, NC).

**Cytological analysis and microscopy**

Microscopic analysis of different developmental stages of the *gfp* and *gfp-ppoA* fused strains were performed with cultures grown for a time course of eight days under dark or light
conditions on solid GMM plates recording the observations on a daily basis. Cells were visualized using an Olympus BX60F-3 fluorescent microscope with a standard FITC filter for GFP and images were captured by an Olympus digital camera (Olympus America Inc.). Nuclei were stained with the DNA-specific dye 4,6-diamino-2-phenylindole (DAPI). Lipid bodies in A. nidulans tissues were visualized by staining with a Nile Red solution (Nile Blue A Oxazone, Signa-Aldrich Co.), containing 1% (w/v) Nile Red in 100% ethanol (32). The tissue fixation was performed as described previously (33). TRITC filter was used to observe the Nile Red fluorescence.

RESULTS

The A. nidulans ppoA gene encodes a protein with oxygenase and peroxidase domains.

A search of the Cereon Genomics A. nidulans database identified a sequence encoding a polypeptide (named ppo gene for psi producing oxygenase) that showed near identity with the homotetrameric ferric hemeprotein, linoleate diol synthase (Lds) from G. graminis (E value: 3x10^{-223}), thus suggesting an identical function for both proteins; the generation of C18 oxylipins. Analysis of A. nidulans genetic maps revealed that the gene is located on chromosome VII. Genomic and cDNA analysis demonstrated that ppoA encodes a 1081 amino acid sequence interrupted by 6 introns of 57 bp, 51 bp, 54 bp, 54 bp, 53 bp and 53 bp respectively. The deduced amino acid sequence of PpoA had significant structural identity with Lds (48% identity, 63% similarity) (34), the previously characterized Ssp1 protein from Ustilago maydis (31% identity, 48% similarity) (33) and the predicted products of several genes from existing filamentous fungal databases. The ppoA gene was not found in Saccharomyces cerevisiae or
Schizosaccharomyces pombe databases. PpoA amino acid sequence also shared high similarity with various mammalian prostaglandin H synthases (PGHs) ranging between 23-24% identity and 40-41% similarity. Like Lds, PpoA contained the distal and proximal His heme ligands and Tyr residue (involved in formation of a tyrosyl radical and ferryl oxygen intermediates during catalysis) shown to be essential for the catalytic activity of mammalian PGHs (34). Conserved domain search of the PpoA protein using NCBI Conserved Domain Search analysis indicated that the residues 210-580 had domains similar to mammalian heme peroxidases and residues 650-1050 had oxygenase domains similar to cytochrome CypX (P450 monoxygenase) (http://www.ncbi.nlm.nih.gov/Structure). Taken together these data suggest that PpoA is a likely ortholog of Lds.

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

To assess the phenotype of a ppoA null mutant (ΔppoA), the gene was inactivated by homologous recombination with pDIT3.11 in strain pW1. PCR and Southern analysis of 138 transformants revealed the replacement of the ppoA gene with the metG gene in four transformants that showed identical phenotypes (data not shown). These transformants had no alterations in radial or vegetative growth compared to the parental strain, however both asexual and sexual development were altered as described below. One transformant, TDIT2.19, was selected for in-depth physiological and molecular analysis. Complementation of the ΔppoA strain with a functional copy of ppoA returned wild type phenotype, thus confirming that the effects on sexual and asexual sporulation were solely due to the deletion of ppoA (data not shown).
PpoA is involved in the production of psiBα.

Fatty acid composition, analyzed from dark grown cultures at 37 ºC, did not significantly differ between wild type, \( \Delta \) ppoA or ppoA overexpression strains (data not shown) with palmitic, oleic and linoleic acids being the most prevalent fatty acids (11, 35). However, measurements of the two most abundant psi factor components, the oleic acid derived 8-hydroxy-9(Z)-octadecanoic acid (8-HOE = psiBβ) and the linoleic acid derived 8-hydroxy-9(Z),12(Z)-octadecadienoic acid (8-HODE = psiBα) (11-13) were statistically different in the three isogenic strains (Table III) resulting in altered 8-HOE/8-HODE ratios that were correlated with changes in the asexual to sexual spore ratios (Table IV). Deletion of the ppoA allele resulted in almost complete elimination of psiBα and a slight increase in psiBβ levels. In contrast, overexpression of ppoA led to a 15 fold and two fold increase in psiBα and psiBβ levels respectively. The presence of linoleic or oleic acid derived psiA or psiC was not detected in any samples in accordance to previous studies (11). These data demonstrated that PpoA is involved in the production of psiB components and suggested that linoleic acid can be a preferable substrate for PpoA.

Deletion of ppoA results in an increase of the asexual to sexual spore ratio; overexpression of ppoA has the opposite effect.

Spore production on GMM, media typically used to produce conidia (11), was measured three and five days after inoculation (Fig. 2). Under light conditions there was a significant increase in conidial production in the \( \Delta \) ppoA strain at day 5 (p<0.01) (Fig. 2A). Under dark conditions \( \Delta \) ppoA produced significantly more conidia than the wild type at both time points (p<0.01) (Fig. 2B) but lower number of ascospores at day 5 compared to wild type (p<0.01) (Fig.
The ratio of asexual spore development to sexual spore development under dark conditions increased approximately four fold in the \( \Delta \text{ppoA} \) mutant after five days of cultivation (Table IV).

Sexual spore production was also assessed for cultures grown on YGT media in the dark, a condition commonly used to promote sexual spore development (9, 10, 31). The number of Hülle cells at day 2 and ascospores at day 4 was not affected by deletion of \( \text{ppoA} \). However, after six days ascospore numbers decreased significantly in the \( \Delta \text{ppoA} \) mutant (\( p<0.01 \)) (data not shown), thus showing similarity to the \( \Delta \text{ppoA} \) strain grown on GMM (Fig. 2).

Next we examined the effect of misscheduled expression of \( \text{ppoA} \) by fusing the \( \text{ppoA} \) gene to the constitutive promoter \( \text{gpdA} \) and to the inducible promoter \( \text{niiA} \). The \( \text{gpdA}(p)::\text{ppoA} \) strain showed a significant increase in sexual spore production and a decrease in asexual sporulation under both light and dark conditions on GMM (Fig. 3A and 3B). These results were maintained over a time period of 10 days (data not shown). In stark contrast to the \( \Delta \text{ppoA} \) phenotype, the ratio of asexual to sexual spore development decreased approximately six fold in the \( \text{gpdA}(p)::\text{ppoA} \) strain after five days of cultivation (Table IV). With the exception of ascospore numbers during growth in dark, similar results were obtained with the nitrogen inducible \( \text{niiA}(p)::\text{ppoA} \) strain (Fig. 3A and 3B). Growth of the \( \text{niiA}(p)::\text{ppoA} \) strain on the \( \text{niiA} \) repressing source ammonia yielded wild-type phenotype (data not shown) indicating that the expression level of \( \text{ppoA} \) is correlated with the increased sexual spore phenotype.

**PpoA localizes in lipid bodies in Hülle cells and metulae.**

The cellular localization of the PpoA protein was determined by constructing a \( \text{ppoA}::\text{green fluorescent protein} \) (GFP) fusion. Microscopic observations revealed GFP
localization only to metulae (Fig. 4B), Hülle cells (Fig. 4C) and initial stages of cleistothecia formation (Fig. 4D). Metulae are mononuclear cells branching from conidiophore vesicles. Conidiogenous cells are borne on metulae (Fig 4A). Hülle cells, typically found surrounding the sexual fruiting bodies (cleistothecia), are large multinucleate cells bearing a thickened cell wall, with characteristic thin-walled pores (Fig. 4A) (36).

During asexual development both GFP and Nile Red dye, specific for lipid bodies (32), were localized to metulae cells (Fig. 4B). During sexual development, PpoA initially localized in Hülle cells formed at the stage of cleistothecial primordium formation (Fig. 4A) and subsequently in immature cleistothecia (12-24h old) as seen in Fig. 4C-4D. Further analyses indicated that the GFP was restricted to distinct globular organelles inside these cells. These organelles were also labeled by Nile Red. DAPI staining and a peroxisome specific GFP-fused protein showed that PpoA was not localized in either the nucleus or the peroxisome (data not shown). In contrast to the \textit{gpdA(p)::ppoA::GFP} strain, the control strain the \textit{gpdA(p):GFP} fusion did not show any specific localization patterns but the fluorescence was diffused in the cytoplasm of hyphae, conidiophores and conidia, Hülle cells and cleistothecia (Fig 4E).

The co-localization of GFP-PpoA fusion protein with the Nile Red stained organelles in the Hülle cells and metulae suggested that PpoA localizes to lipid bodies. In support of this case, analysis of the amino acid sequence of PpoA indicated that it contains a conserved hydrophobic subdomain known as the “proline knot” that is characteristic for targeting plant proteins to lipid bodies (Fig. 5) (37, 38). This motif is also found in Lds and Ssp1 proteins, the latter shown also to be a spore specific lipid body protein in \textit{U. maydis} teliospores (33).
**ppoA expression is developmentally regulated.**

Considering the phenotype of the *ppoA* deletion and overexpression strains as well as the localization of PpoA, we hypothesized that *ppoA* mRNA would be developmentally regulated. *ppoA* transcript was analyzed from asexually and sexually induced wild type cultures. As depicted in Figure 6A, upon switching from mycelial growth to asexual spore inducing conditions (Panel A), *ppoA* was highly expressed at 6h and 12h, tapering off at 24h, the time of conidiophore maturity in light. *ppoA* expression was more uniform in sexual spore inducing conditions (Fig. 6A, Panel B), although showed a slight peak at 6h and 12h dark conditions, the stage of Hüle cell aggregation. *ppoA* transcript accumulation was not affected by light or dark conditions with the exception of the zero hour time point in the sexual induction conditions (Fig. 6A, Panel B). These results led to the conclusion that *ppoA* expression is correlated with the initiation of asexual and sexual fruiting body formation in *A. nidulans*.

**VeA regulates ppoA expression and is important for PpoA regulation of asexual and sexual sporulation.**

Considering the importance of VeA in sexual and asexual development, we speculated that *veA* allele would affect *ppoA* expression and/or PpoA activity. Therefore, we compared the expression of the *ppoA* gene in *veA* strain to *veA1* and Δ*veA* mutants. As shown in Figure 6B, *ppoA* transcripts were elevated in the *veA1* strain under vegetative growth conditions and the early asexual developmental stages (Panel A) but repressed when the fungus entered the sexual developmental program (Panel B). *ppoA* expression was virtually eliminated in the Δ*veA* strain (Fig. 6C, Panels A and B).
Further evidence of a PpoA/VeA interaction was observed by comparing the ΔppoA; veA and ΔppoA; veA1 phenotypes. Although both strains showed an increase in conidia production compared to their respective ppoA wild type (p<0.01) (Fig. 2A, 2B and Fig. 7A, 7B), the ΔppoA; veA1 strain also showed an increase in ascospore production in the dark in contrast to the ΔppoA; veA strain (Fig. 2C, 7C). Ascospore production is inhibited in the veA1 genotype and this may suggest that inhibition requires PpoA activity in this genetic background. Deletion of ppoA in the ΔveA background did not alter the phenotype of a ΔveA strain (data not shown). Taken together, these data demonstrate that VeA is required for normal ppoA expression and that VeA/PpoA interactions affect sexual and asexual development.

**ppoA expression is regulated by the COP9 signalosome.**

Psi factor was originally isolated and chemically characterized from the psi overproducing acoC and acoB mutants, WIM145 (acoC193) and WIM146 (acoB202) respectively (8). AcoB and AcoC3 show high amino acid sequence similarity to components of the plant and mammalian multiprotein complex COP9 signalosome (CSN) (39). The CSN complex is conserved from fission yeast to humans and functions in various physiological processes such as cell cycle, transcriptional control, light- and hormone-dependent pathways (40). Transcript analysis showed that ppoA expression is misscheduled and upregulated in both the acoC193 and acoB202 mutants (Fig. 6D, Panels A and B and data not shown), thus correlating ppoA expression with psi over production in these strains.
DISCUSSION

A striking aspect of morphogenesis in many filamentous fungi is the presence of both an asexual and a sexual spore reproductive cycle. Due to the importance of fungal spores in initiating a plant or mammalian infection, establishing a fungal colony and dissemination as well as the importance of sexual fruiting bodies as overwintering structures and aids in genus and species identification, there has been considerable interest in identifying factors that regulate fruiting body and spore formation. In our studies we are focusing on identifying endogenous signals that can integrate and balance anamorph and teleomorph development. Here we describe an *A. nidulans* lipid body protein, PpoA, a putative oxylipin producing oxygenase that regulates the asexual to sexual spore ratio.

**PpoA is involved in the production of the linoleic acid derived oxylipin psiBα.**

Chemical analysis of ∆ppoA and OE::ppoA strains demonstrated that deletion of *ppoA* did not alter the major fatty acid profile in *A. nidulans*. However, the ∆ppoA mutant was crippled in its ability to synthesize psiBα (Table III), otherwise known as 8-HODE, the same metabolite synthesized by *G. graminis* Lds (14). Additionally, the amount of psiBα detected in the overexpression *ppoA* strain was ca. 15 fold greater than that of wild type (Table IV). Slight increases in psiBβ (8-HOE) content, the oleic acid derived psi moiety, in ∆ppoA and OE::ppoA strains possibly indicated that another enzyme responsible for the oxygenation of oleic acid was upregulated in both mutants. However, we can not exclude the possibility that oleic acid can also act as a minor substrate for PpoA since 8-HOE was increased approximately two fold in the OE::ppoA strain. The fact that the other components of psi factor, psiC and psiA were not detected indicates that either these compounds are in low undetected levels, or are unstable.
PsiCβ was previously identified in a mutant strain that accumulates significant amounts of oleic acid (11). In the case of psiA, we can not exclude the possibility that it is an artifact of the extraction methodology that used for its characterization (12, 13). Further biochemical analyses will clarify the actual substrates of the *ppoA* in vitro.

**PpoA activity increases sexual sporulation and decreases asexual sporulation.**

Deletion of *ppoA* led to a four fold increase in the asexual to sexual spore ratio, whereas overexpression of *ppoA* led to a six fold decrease in the asexual to sexual spore ratio (Fig. 2, 3 and Table IV). These results suggest that the PpoA product(s) act as positive regulator(s) of ascosporogenesis and/or negative regulator(s) of conidiation. Champe *et al.* (10) observed that exogenously applied psiBα induces premature cleistothecia formation and sexual sporulation and inhibits conidiation, which are characteristics of the *OE::ppoA* phenotype. This suggests an important role for psiBα in the Δ*ppoA* and *OE::ppoA* phenotypes. It is also possible that the phenotypic changes were due to the altered ratio of psiBα to psiBβ in the mutants (Table IV) or even downstream derivatives of psiB oxylipins or any combinations thereof. For instance, Calvo *et al.* (11) noted a correlation between increasing psiBβ content and ascospore formation, as we also observed in the Δ*ppoA* strain. Interestingly, exogenously applied seed linoleic acid derived oxylipins also alter the asexual to sexual spore ratio in wild type *A. nidulans* (31). Together these studies strongly support a role for oxylipins in maintaining a balance between sexual and asexual spore development in *A. nidulans*. Considering that putative homologs exist in all the filamentous fungi examined, it is possible that this phenomenon is conserved in other fungi. Deletion of Ssp1, a protein that is predominantly expressed in mature teliospores of *U. maydis* led to no altered phenotype, however assessment of spore production was not reported (33).
PpoA is a lipid body protein and localizes in fruiting bodies.

Microscopic studies revealed that PpoA co-localized with organelles stained by Nile Red, a dye predictive of lipid bodies (Fig. 4). Lipid bodies are macromolecular proteolipid assemblies that contain neutral lipids important as storage fats and oils (41, 42). The lipid bodies’ surface proteins probably play a role in lipid-body biogenesis, trafficking, mobilization and metabolism (43, 44). Lipid body proteins include prostaglandin H synthases in mammalian tissues (45), oleosin (38), caleosin and isoforms of lipoxygenases (46) in plant tissues and Ssp1 in teliospores of U. maydis (33). Previous studies have also demonstrated that the plant oleosin protein is targeted to the lipid bodies in a transformed yeast strain indicating that the lipid body localization motifs are conserved between plants and fungi (47). As shown in Figure 5, Ssp1, Lds and PpoA all contain the hallmark proline knot motif indicative of lipid body targeting. Taken together these studies support a model for the presence of PpoA in lipid bodies.

Lipid bodies are commonly found in fungal tissues particularly during the formation of resting and reproductive structures (42, 44). Our studies supported these observations by identifying high concentrations of lipid bodies in the metulae and Hülle cells in A. nidulans. The presence of lipid bodies in these tissues might be important for PpoA effects on conidial and ascospore development.

PpoA is expressed during the formation of fruiting bodies.

Our GFP studies indicated that the location of PpoA is highly correlated with its target tissues, conidiophores and cleistothecia. Figure 6A (Panels A-B) shows that ppoA expression was also associated with formation of these fruiting bodies. This correlation suggested that PpoA activity is important over time and space for normal sporulation processes in A. nidulans.
Constitutive expression of \textit{ppoA} led to precocious sexual development and to delayed asexual spore formation, phenomena that were also correlated with the observed significant increase in psiB production. Examination of the putative promoter region of \textit{ppoA} revealed the presence of binding domains for several known fungal (e.g. \textit{stuA}) and mammalian (e.g. SREBP-1) transcription factors suggesting that the transcriptional regulation of \textit{ppoA} is under the control of complex cellular molecular pathways. Huber \textit{et al}. (33) analyzed the 5’ region of the \textit{ssp1} gene and by deletion studies identified undefined promoter regions that are essential in maintaining the gene in a repressed state during saprophytic growth as well as an element that appears to be necessary for stage-specific regulation or induction in spores.

\textbf{PpoA is regulated by veA.}

The sporogenic response to linoleic acid moieties requires the presence of an intact \textit{veA} gene in \textit{A. nidulans} (31). The \textit{veA} gene is known to have a role in activating sexual development and/ or inhibiting asexual development (5-7). Two lines of evidence (Fig. 6B, 6C) suggested that \textit{veA} regulates directly or indirectly the transcription of \textit{ppoA} gene: (i) in \textit{veA1} mutants \textit{ppoA} was expressed at very high levels only during asexual development and (ii) deletion of \textit{veA} caused inhibition of \textit{ppoA} transcript accumulation. \textit{veA1} mutants are sexually defective; however disruption of \textit{ppoA} in \textit{veA1} background led to wild type level ascospore production suggesting that the early high level expression of \textit{ppoA} in this mutant might account for the significant increase in conidia production and decrease in ascospore development (Fig. 7). The absence of \textit{ppoA} transcripts in \textit{\Delta veA} strains might also be correlative with the sexual defects in this strain.

\textbf{The COP9 signalosome regulates \textit{ppoA} expression.}
Psi factor was first identified in \textit{acoB202} and \textit{acoC193} mutants, pleiotrophic strains where psi compounds are over produced (8, 10). Figure 6D (Panels A-B) shows that \textit{ppoA} was upregulated in the \textit{acoC193} strain, thus indicating that AcoC negatively regulates \textit{ppoA} expression. Similar results were obtained examining the \textit{acoB} mutant (data not shown). The amino acid sequence of AcoB (GenBank Accession # U18265) reveals that it has high similarity to the \textit{Arabidopsis} CSN7 protein and AcoC \textsuperscript{3} protein is identical to the cloned \textit{A. nidulans csnD} gene (39). CSN7 and CSND are components of the COP9 signalosome, the eight-component complex found in all multicellular eukaryotes (40). Recent data indicate that the \textit{A. nidulans} COP9 signalosome is a key regulator of light dependent signaling and asexual and sexual development (39). Based on our data we speculate that the \textit{Aspergillus} COP9 multiprotein complex controls the transcription factors that regulate the expression and/or transcript degradation of \textit{ppoA} and as a consequence the production of the corresponding oxylipin products. The elevated levels of oxylipins in \textit{acoB202} and \textit{acoC193} strains may account for some of the developmental defects in these mutants.

In conclusion we have characterized the \textit{ppoA} gene, which is likely responsible for the production of the sporogenic psi factor psiB\textalpha. PpoA was required for integrating asexual and sexual spore development, a phenomenon that can be directly attributed to the oxylipin composition in the fungal cell. The developmental regulators VeA and the COP9 signalosome affect the expression level of \textit{ppoA} and reception of the psi signal (31) suggesting the presence of a cascade of molecular circuits in the fungal thallus that ultimately lead to the integration of the development of the anamorphic and teleomorphic stages. We hypothesize that oleic and linoleic derived oxylipins are conserved regulators of meiotic and mitotic spore balance in filamentous fungi.
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FOOTNOTES

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1 The abbreviations used are: GMM, glucose minimum medium; bp, bases; FAME, fatty acid methyl esters; FID-GC, flame ionization detection-gas chromatography; OTMSi, trimethylsilyloxy; PUFA, polyunsaturated fatty acids; GC-MS, gas chromatography-mass spectrometry; GC-EI.MS, gas chromatography-electron impact/mass spectrometry; 8-HOE, 8-hydroxy-9-octadecanoic acid; 8-HODE, 8-hydroxy-9,12-octadecadienoic acid.

2 J-W. Bok and N. P. Keller, unpublished data.

3 K. Sutton and L. Yager, personal communication.

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LEGENDS TO FIGURES

Figure 1: Chemical structures of the *Aspergillus nidulans* linoleic acid derived psi factor components.

Figure 2: Deletion of *ppoA* increases conidial production and decreases ascospore production. Cultures of wild type (RDIT9.32) and Δ*ppoA;veA* (RDIT12.9) were grown at 37 °C under dark and light conditions in Glucose Minimum Medium (GMM). *Panel A*, conidia production of 3 and 5 day-old cultures grown in GMM in light. *Panel B*, conidia production of 3 and 5 day-old cultures grown in GMM in dark. *Panel C*, ascospore production of 5 day-old culture grown in GMM in dark. 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<0.01).

Figure 3: Overexpression of *ppoA* decreases conidial production and increases ascospore production. Cultures of wild type (RDIT9.32), *gpdA(p)::ppoA* (RDIT81.10) and *niiA(p)::ppoA* (RDIT75.24) were grown at 37 °C under dark and light conditions on Glucose Minimum Medium (GMM) for five days. *Panel A*, conidia production. *Panel B*, ascospore production. 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<0.01).

Figure 4: PpoA localizes in lipid bodies of asexual and sexual fruiting bodies. Strains RDIT71.8 and TDIT9.14 were grown on solid GMM. *Panel A*, terminology of the structures of a mature conidiophore (a) and a young cleistothecium surrounded by Hülle cells (b) of *Aspergillus nidulans* (drawings were taken with permission from ref. 48). *Panel B*, isolated conidiophores were observed under light microscopy (a, c) and fluorescence microscopy (b, d). The
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conidiophore of the picture “b” depicts the PpoA localization in lipid bodies that are stained red with the dye Nile Red (d). *Panel C*, individual Hülele cells observed under light microscopy (a), fluorescence microscopy for GFP (b) and Nile Red staining (c). PpoA localizes in lipid bodies in Hülele cells. *Panel D*, young cleistothecium surrounded by Hülele cells as seen by light microscopy (a) and fluorescence microscopy (b). PpoA localizes in the young cleistothecium at this developmental stage. *Panel E*, hyphae (a) and Hülele cells (b) of the control *gpdA* (p)::*gfp* strain (TDIT9.14) observed under fluorescence microscopy show cytoplasmic localization of the GFP.

**Figure 5: Partial amino acid alignment of the proline knot motif with PpoA, Lds and Ssp1 sequences.** The proline knot is an essential motif for targeting proteins to lipid bodies. PpoA: Psi Producing Oxygenase from *A. nidulans*, Lds: Linoleate Diol Synthase from *G. graminis* and Ssp1: Spore Specific Protein from *U. maydis*. Identical amino acids are marked with “*” and similar amino acids are marked with “:”. The conserved proline residues are in bold. The alignment was performed with ClustalW.

**Figure 6: ppoA is transcriptionally regulated under asexually and sexually differentiated cultures.** Transcript analysis of *ppoA* in (A) wild type veA (RDIT9.32), (B) veA1 (RDIT2.3), (C) ΔveA (RDIT47.37) and (D) acoC, veA (RDIT19.12) RNA isolated at different time points during development. Mycelia grown for 18h in liquid GMM were collected and transferred onto solid GMM media (time 0). Induction of asexual sporulation was performed under normal aeration conditions and at appropriate time intervals samples were collected for RNA analysis (6h to 48h) under dark or light conditions. The time point “–2” corresponds to 16h vegetative growth culture, 2h before the transfer to solid media. For the induction of sexual sporulation 18h liquid grown mycelia transferred onto solid media and the plates were sealed with parafilm for
20h under dark or light conditions. After 20h the plates were unsealed and at different time points samples were collected for RNA analysis (time represents hours after induction: 0h-36h). The time point “T” corresponds to the sample that was collected at the time of transfer to the solid media and before the sexual induction. Equal loading of total RNA (20 µg) is depicted by ethidium bromide staining of the rRNA. The time points of harvesting are indicated above the lanes.

**Figure 7: Deletion of ppoA in veAl background increases conidial and ascospore production.** Cultures of the isogenic strains veAl (RDIT2.3) and ΔppoA; veAl (RDIT12.1) were grown at 37 ºC under dark and light conditions on YGT media for six and eight days. **Panel A,** conidia production of cultures grown in light. **Panel B,** conidia production of cultures grown in dark. **Panel C,** ascospore production of cultures grown in dark. Values are the means 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<0.01).
### TABLE I

*Aspergillus nidulans* strains used in this study

| Fungal strain | Genotype                                                   | Source       |
|---------------|------------------------------------------------------------|--------------|
| pW1           | biA1; argB2; metG1, veA1                                   | FGSC<sup>a</sup> |
| FGSC773       | pyrG89; pyroA4; wA3; veA1                                  | FGSC         |
| WIM126        | pabaA1; yA2; veA                                          | L. Yager     |
| FGSC237       | pabaA1; yA2; veA1; trpC801                                | FGSC         |
| FGSC33        | biA1; pyroA4; veA1                                         | FGSC         |
| WIM145        | pabaA1; yA2; acoC193; veA                                  | L. Yager     |
| WIM146        | pabaA1; yA2; acoB202; veA                                  | L. Yager     |
| DVARI         | pabaA1; yA2; ΔargB::trpC; ΔveA::argB; trpC801             | (4)          |
| RDIT1.1<sup>b</sup> | pyrG89; argB2; metG1; veA1                              | This study   |
| RDIT1.7       | biA1; metG1; veA1                                         | This study   |
| RRAW8         | pyrG89; biA1; argB2; metG1; veA1                          | R.A. Wilson  |
| RDIT30.32     | pyrG89; argB2; metG1; veA1; trpC801                        | This study   |
| RDIT12.3      | biA1; argB2; metG1; ΔppoA::metG; veA                       | This study   |
| RDIT44.10     | pabaA1; biA1; pyroA4; metG1; veA1; trpc801                 | This study   |
| RDIT2.30      | metG1; veA                                               | This study   |
| TDIT3.10<sup>b</sup> | biA1; argB2; metG1::metG, veA1                              | This study   |
| TDIT2.9       | biA1; argB2; metG1; ΔppoA::metG; veA                       | This study   |
| RTMK22.28     | biA1; pyroA4; metG1; ΔppoA::metG; veA1; trpC801          | T.M. Kowieski |
| TDIT8.1       | biA1; ppoA::pyroA; metG1; ΔppoA::metG; veA1; trpC801      | This study   |
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veA1; trpC801

**Prototrophic isogenic strains**

| Strain Code | Description | Source |
|-------------|-------------|--------|
| RDIT30.92   | argB2; metG1; veA1; trpC801 | This study |
| RDIT30.12   | pyrG89; argB2; veA1; trpC801 | This study |
| TDIT4.2     | argB2; metG1; veA1; niiA(p)::ppoA::trpC | This study |
| TDIT5.8     | pyrG89; argB2; veA1; gpdA(p)::ppoA::trpC | This study |
| RTMK22.16   | biA1; pyroA4; metG1; ΔppoA::metG; veA; trpC801 | T.M. Kowieski |
| RDIT30.23   | argB2; metG1; veA1; trpC801 | This study |
| TDIT7.3     | argB2; metG1; veA1; gpdA(p)::gfp::ppoA::trpC | This study |
| RDIT12.15   | biA1; metG1; ΔppoA::metG; veA | This study |
| RDIT55.27   | pyroA4; metG1; veA; trpC801 | This study |
| RDIT55.31   | pyrG89; pyroA4; veA; trpC801 | This study |
| TDIT9.14    | gpdA(p)::gfp::pyr4; pyroA4; veA; trpC801 | This study |
| RDIT9.32    | veA | This study |
| RDIT2.3     | veA1 | This study |
| RDIT16      | metG1::metG; veA | This study |
| RDIT19.12   | acoC193; veA | This study |
| RDIT47.37   | ΔargB::trpC; ΔveA::argB; trpC801 | This study |
| RDIT12.1    | metG1; ΔppoA::metG; veA1 | This study |
| RDIT12.9    | metG1; ΔppoA::metG; veA | This study |
| RDIT83.1    | metG1; ppoA::pyroA; ΔppoA::metG; veA | This study |
| RDIT83.2    | metG1; ppoA::pyroA; ΔppoA::metG; veA1 | This study |
| RDIT75.24   | metG1; ΔppoA::metG; veA; niiA(p)::ppoA::trpC | This study |
| Strain    |Description                          | Source          |
|----------|-------------------------------------|-----------------|
| RDIT81.10| \( \text{metG}\); \(\Delta ppoA::\text{metG}\); veA; gpdA(p)::ppoA::trpC | This study      |
| RDIT71.8 | \( \text{metG}\); \(\Delta ppoA::\text{metG}\); veA; gpdA(p)::gfp::ppoA::trpC | This study      |

\(^a\) FGSC: Fungal Genetics Stock Center, Kansas City, KS, USA

\(^b\) Strains starting with “T” are original transformants and “R” are recombinants after sexual cross. Some of the strains are not described in the text but were used for sexual crosses to create the final prototrophic strains.
### TABLE II

**Oligonucleotides used in this study**

| Oligonucleotide | Sequence*                  | Restriction Enzyme Site* |
|-----------------|----------------------------|--------------------------|
| ppoA-F1         | 5’-GCCGATTCACCAAAACCTG-3’ | —                        |
| ppoA-R1         | 5’-CGGATGAGAGATACGACTG-3’ | —                        |
| ppoA-F2         | 5’-GATGGGTGAAGACAAAG-3’   | —                        |
| ppoA-R2         | 5’-CGGGGTATTTTCTTTGG-3’   | —                        |
| ppoA-5DF3       | 5’-CTGGGGTTGTCTTAGAGTGAACTATG-3’ | XbaI                |
| ppoA-5DR1       | 5’-CATCGTGAACCCGGGGCTTTATAG-3’ | XmaI                |
| ppoA-3DF1       | 5’-GGAGCAGTTGCATGCAAGGTGCTC-3’ | SphI                |
| ppoA-3DR1       | 5’-GGTGATGGGGGTACCATAGCTTG-3’ | KpnI                |
| ppoA-F15-KpnI   | 5’-AGCTTTTCGTTGATACCTCTTCTCCATC-3’ | KpnI                |
| ppoA-R18-KpnI   | 5’-GGCTAGTGCTAGGTACCTGGC-3’ | KpnI                |
| gpdA-5-NotI     | 5’-TTATGGCTTTG CGGCCGCTATGTTGTGGTAATG-3’ | NotI                |
| gpdA-3-NcoI     | 5’-TCTAGCTTTCCATGGGTATGTCTG-3’ | NcoI                |
| NGPPOA-5’GFP    | 5’-CTAGCGGAAGGT ACCCATGACGCAAGGGCG-3’ | KpnI                |
| NGPPOA-3’GFP    | 5’-AGTGCCGCACGCTTGCTACAG-3’ | SphI                |
| NGPPOA-5’ppoA   | 5’-ATACTATCTGCACTGTTCTGTA-3’ | SphI                |

* Underlined sequences show the introduced restriction enzyme site in the corresponding primer.
TABLE III  
Psi factor composition of mycelia of wild type, ΔppoA and gpdA(p)::ppoA strains

| Sample          | Hydroxy-FAME µg/g mycelium (dry weight) |
|-----------------|-----------------------------------------|
|                 | 8-HOE (psiBβ)                           | 8-HODE (psiBα) |
| Wild type       | 5.87 ± 0.70c                             | 2.19 ± 0.87    |
| ΔppoA           | 7.70 ± 0.69*                            | 0.22 ± 0.19**  |
| gpdA(p)::ppoA   | 14.45 ± 3.36*                           | 35.17 ± 2.15** |

The analysis was carried out with 72h old mycelia grown in liquid glucose minimum media under stationary conditions at 37 °C. Statistical analysis was performed by using Student t-test and significance is indicated as follows: *, p<0.05 and **, p<0.001.

8-HOE: 8-hydroxy oleic acid = psiBβ; 8-HODE: 8-hydroxy linoleic acid = psiBα.

Values are the means of three replications ± S.E.
TABLE IV

Ratios of oleic to linoleic acid derived psi factor and asexual to sexual spore production

| Strain             | 8-HOE : 8-HODE \(^{a,b}\) | Asexual : Sexual \(^a\) |
|--------------------|---------------------------|-------------------------|
| Wild type          | 2.7 : 1                   | 1 : 1.2                 |
| \(\Delta ppoA\)   | 35 : 1                    | 3.1 : 1                 |
| gpdA(p)::ppoA      | 1 : 2.4                   | 1 : 6.7                 |

\(^a\) The ratios of 8-HOE to 8-HODE and of asexual to sexual spore production (spores/mm\(^2\)) were calculated for each strain under dark conditions after five days growth on solid GMM.

\(^b\) 8-HOE: 8-hydroxy oleic acid = psiB\(\beta\); 8-HODE: 8-hydroxy linoleic acid = psiB\(\alpha\)
Figure 1
Figure 2

A

\[
\text{Conidia (x10^3/mm^2)}
\]

\[
\begin{array}{c|c|c}
\text{Days after inoculation} & \text{LIGHT} & \text{DARK} \\
\hline
3 & 350 \pm 10 & 200 \pm 10 \\
5 & 600 \pm 20 & 300 \pm 15 \\
\end{array}
\]

Days after inoculation (GMM)

B

\[
\text{Conidia (x10^3/mm^2)}
\]

\[
\begin{array}{c|c|c}
\text{Days after inoculation} & \text{LIGHT} & \text{DARK} \\
\hline
3 & 150 \pm 5 & 100 \pm 5 \\
5 & 400 \pm 10 & 250 \pm 15 \\
\end{array}
\]

Days after inoculation (GMM)

C

\[
\text{Ascospores (x10^3/mm^2)}
\]

\[
\begin{array}{c|c|c}
\text{Days after inoculation} & \text{DARK} \\
\hline
5 & 100 \pm 5 & 30 \pm 2 \\
\end{array}
\]

Days after inoculation (GMM)

- **Wild type**
- **Δppoa**

*Significant difference*
Figure 3

A

Conidia (x10^3/mm^2)

DARK LIGHT

B

Ascospores (x10^3/mm^2)

Wild type

gpdA(p)::ppoA

niiA(p)::ppoA

DARK LIGHT

*
Figure 5

| Protein    | Sequence                        | Position |
|------------|---------------------------------|----------|
| PpoA       | AFPNHDGNSIYAHFPLVPS             | 627      |
| Ssp1       | TLPGQFPYNISIYALYFRVPE           | 629      |
| Lds        | AFPNHFLPNSYAHFPLVPS             | 629      |
| Proline knot | ATP-LF---VIF---PVLVPA         |          |

Figure 6

| Panel A | Panel B |
|---------|---------|
| **DARK** | **DARK** |
| -2 0 6 12 24 36 48 | -2 0 6 12 24 36 48 |
| **LIGHT** | **LIGHT** |
| 6 12 24 36 | 0 6 12 24 36 |

**Figure 5**

**Figure 6**

**Panel A**

-2 0 6 12 24 36 48

**Panel B**

0 6 12 24 36

ASEXUAL CONDITIONS

SEXUAL CONDITIONS
Figure 7

A

B

C

Days after inoculation (YGT media)

Days after inoculation (YGT media)

Days after inoculation (YGT media)

Conidia ($10^3$ / mm$^2$)

Conidia ($10^3$ / mm$^2$)

Conidia ($10^3$ / mm$^2$)

Ascospores ($10^3$ / mm$^2$)

Ascospores ($10^3$ / mm$^2$)

Ascospores ($10^3$ / mm$^2$)

veA1

ΔppoA, veA1

veA1

ΔppoA, veA1

veA1

ΔppoA, veA1

veA1

ΔppoA, veA1
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Dimitrios I. Tsitsigiannis, Robert Zarnowski and Nancy P. Keller

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