Signaling events connecting mycotoxin biosynthesis and sporulation in *Aspergillus* and *Fusarium* spp.

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Summary

Secondary metabolite production and sporulation are tightly co-regulated in the Aspergilli and Fusaria. Here we discuss two conserved pathways, including a G-protein/cAMP/Protein kinase A cascade and an oxylipin-mediated signalling process, that genetically link sporulation and secondary metabolism in these genera. The G-protein alpha subunit FadA negatively regulates aflatoxin and cyclopiazonic acid production in *Aspergillus*, and positively regulates penicillin production in *A. nidulans* and tricothecene production in *Fusarium sporotrichiodes*. Inactivation of FadA eliminates or decreases asexual spore production in both genera. The G-protein cascade is conserved throughout eukaryotes, and regulation of sporulation and secondary metabolism by this signal transduction pathway appears to be conserved within filamentous fungi. On the other hand, the oxylipin-mediated signalling pathway appears to be restricted to filamentous fungi. We have identified novel genes encoding putative dioxygenases likely to be responsible for secreted oxylipins which act as sporulation factors. Deletion of these genes affects asexual sporulation and secondary metabolite production in *A. nidulans* and *F. sporotrichiodes*.

Key words : toxins, sporulation, G-protein, oxylipin, secondary metabolites

Introduction

Secondary metabolites synthesized by fungi and other organisms are a varied group of low molecular weight natural products that are of intense interest to humans due to their myriad beneficial applications in medicine and industry as well as their detrimental effects on human health. Some metabolites have antibiotic, antiviral, or anti-tumor properties. Others are valued by industry as pigments, volatiles, or fermentation products. Many are implicated in disease of crop plants, livestock and humans, and may be carcinogenic or toxic. Examples of most of these properties may be found in
Aspergillus species. Aspergillus nidulans is a valuable model system for studying the production of secondary metabolites for several reasons. Although not a pathogen itself, it is closely related to two common plant pathogens of major crop species A. flavus and A. parasiticus, the human pathogen A. fumigatus as well as the industrial food fermentator A. oryzae. All of these species are reported to contain the gene cluster for aflatoxin (AF), a toxic and carcinogenic contaminant of grain and oilseed crops. In A. nidulans, the pathway is terminated at the penultimate step producing sterigmatocystin (ST), a mycotoxin with similar properties as AF. A. nidulans also contains the penicillin (PN) gene cluster. Our understanding of fungal secondary metabolism has been greatly aided by investigations of ST and PN biosynthesis in this fungus. A. nidulans has proven to be an excellent model system as, unlike other Aspergilli, it has a sexual cycle, which makes it genetically tractable. Additionally, a genome sequence is available for this organism (http://www-genome.wi.mit.edu/annotation/fungi/), facilitating comparative genomics with other sequenced Aspergilli, including A. flavus, A. fumigatus, and industrial species A. niger and A. oryzae, thus making the Aspergilli prime targets for research of all aspects of fungal development. Moreover, genetic mechanisms elucidated in A. nidulans have been shown to be conserved not only within the genus and within the filamentous fungi, but also throughout the fungal kingdom and in some cases in mammalian systems.

The ease with which Aspergillus nidulans is manipulated facilitates the elucidation of genetic mechanisms controlling secondary metabolism in less tractable genera, such as Fusarium. Although Aspergillus and Fusarium are the two most prevalent mycotoxic genera worldwide and frequently contaminate many of the same crop species, sometimes simultaneously, comparatively less work has been done on the genetics of secondary metabolism in Fusarium partly because Fusaria are more difficult to manipulate. But advances in A. nidulans have elucidated mechanisms regulating mycotoxins and antibiotics in Fusarium. In general the regulatory elements, although not necessarily their effects, are highly conserved between the genera.

We focus here on the co-regulation of secondary metabolite production - with emphasis on mycotoxins - and sporulation in these genera. In all cases, sporulation and secondary metabolite production are intimately connected, both indirectly and directly through common signaling pathways. Two pathways we discuss in detail are a G-protein/cAMP/protein kinase signal transduction relay common to many eukaryotes, and an oxylipin mediated signaling process that may be unique to filamentous fungi.

A G-protein/cAMP/protein kinase A cascade genetically links sporulation and secondary metabolism

Background Previous work in this and other labs has established the importance of
the G-protein signal transduction pathway for both sporulation and secondary metabolite production in Aspergillus nidulans. Heterotrimeric G-protein transduction cascades are conserved in eukaryotes. The G-proteins central to the cascades consist of alpha, beta, and gamma subunits. The beta and gamma units are permanently bound to each other. The alpha unit may be bound to the beta/gamma dimer and to GDP, in which case it is inactive. Or it may be bound to GTP, in which case it is free of the G-protein beta-gamma dimer and is active. Intrinsic GTPase activity of alpha subunit results in the conversion of the GTP-form to the GDP-form and consequent re-association with the beta/gamma subunits. This intrinsic GTPase activity is stimulated by so-called RGS (regulation of G-protein signaling) proteins, also known as GAP proteins. By activating alpha unit GTPase activity, RGS proteins cause it to be locked in the GDP-bound trimer form.

GTP-bound alpha units transmit stimuli by either stimulating or inhibiting adenylyl cyclase activity. Activation of adenylyl cyclase increases the pool of the secondary messenger cAMP available in the cell. The increase in cAMP levels in the cell results in activation of the cAMP-dependent protein kinase (PKA), composed of two regulatory subunits and two catalytic subunits, by freeing the catalytic subunits from the regulatory domains. The catalytic subunits inactivate or activate target proteins by phosphorylation. This pathway is conserved in Aspergilli, in filamentous fungi including Fusarium, and in fungi in general including the yeast Saccharomyces and in mammalian systems\(^2\) (and references therein).

In A. nidulans, several components of the G-protein pathway have been characterized\(^3\). Here we will focus primarily on fadA, encoding the alpha subunit of a heterotrimeric G-protein complex and pkaA, encoding the catalytic subunit of protein kinase A (Fig. 1).

**FadA regulates mycotoxin production in A. nidulans, A. parasiticus, A. flavus and F. sporotrichiodes** Dominant activated FadA alleles, like FadA\(^{G42R}\), have been useful in assessing the downstream effects of FadA\(^a\). These activated alleles of FadA are formed when the regions responsible for inherent GTPase activity are mutated and the protein is locked in the GTP-bound free state.

We have previously shown that activated FadA negatively regulates sterigmatocystin

**Fig. 1.** A model of G-protein regulation of ST and PN production in Aspergillus nidulans.
production in *A. nidulans*, aflatoxin production in *A. parasiticus* and sporulation in both spp.\(^4\) New data show that FadA also represses mycotoxin production in *A. flavus*. The dominant activated *fadA* allele *fadAG42R* was transformed into an aflatoxin and cyclopiazonic acid (CPA) producing *A. flavus* strain. Transformants carrying the *fadAG42R* allele could not produce AF or CPA in contrast to FadA wild-type strains (Fig. 2). These results suggest that FadA negatively regulates production of both mycotoxins in *A. flavus*. In addition to inhibiting mycotoxin biosynthesis in the Aspergilli, FadA also suppresses asexual spore production. Similarly to *A. nidulans* and *A. parasiticus* strains containing the *fadAG42R* allele, the *A. flavus fadAG42R* strain could not produce conidia (asexual spore) (data not shown)\(^4\).

Although both mycotoxin production and sporulation are negatively regulated by FadA in the Aspergilli, two secondary metabolites have been found to be up-regulated in *fadAG42R* strains. Penicillin production is increased in *fadAG42R* strains of *A. nidulans* and trichothecene production is increased in *fadAG42R* strains of *F. sporotrichiodes*\(^5\). However, the repressive effect of *fadAG42R* on conidial production was conserved in *F. sporotrichiodes*.

**Mediation of FadA signaling through PkaA** In *A. nidulans pkaA* has been shown to negatively regulate aflR, the transcription factor regulating AF and ST production, and brlA, the transcription factor regulating asexual sporulation (Fig. 1)\(^2\). Accordingly, overexpression of pkaA mimics the *fadAG42R* phenotype. Deletion of pkaA in a *fadAG42R* genetic background did not restore ST production but partially restored sporulation thus showing that some FadA signaling is transduced through PkaA. However, involvement of pkaA in regulation of PN production in *A. nidulans* was not determined.

Because PkaA transduces the FadA signal in sporulation, we thought PkaA might also transduce the FadA signal in positively regulating PN. However, as shown in Fig. 3, the expression of ipnA (a PN biosynthetic gene) was reduced in a pkaA overexpression background. Examination of ipnA expression in the pkaA deletion background was delayed (data not shown), however PN bioassays showed that PN was still produced in this strain (data not shown). These results suggest that PkaA has little direct effect on PN production. Yet *ipnA* expression in the strain carrying both a pkaA deletion and an activated *fadA* allele was reduced compared to either single mutant (data not shown). This might suggest that FadA signaling of *ipnA* expression is partially transduced by PkaA. The results could also reflect the fact that the double mutant is very sick and cannot grow as well as the single mutants.

**Uncoupling of sporulation and secondary metabolism by a downstream component of the G-signaling pathway** Extensive studies of *A. nidulans* strains carrying mutant *fibA, fadA* and *pkaA* alleles all support a genetic connection of regulation of both sporulation and secondary metabolism. Most recently, however, a novel gene termed *laeA* for loss of aflR expression has been found that regulates secondary metabolism but
Fig. 2. AF and CPA analysis by TLC.
The TLC plate was developed in chloroform-acetone (85:15, v/v) for AF, and in ethyl acetate/methanol/ammonium hydroxide (85:15:10, v/v/v) for CPA. *A. flavus* 12S (lanes 8 through 10) and RAWM1 (lanes 5 through 7) produced AF and CPA, whereas fad\(_{42}^{G42R}\) transformant TSCS1 (lanes 1 through 4) did not. Lane 1 is AF and CPA standard. RAWM1 is a niaD mutant of *A. flavus* 12S. *A. flavus* TSCS1 was generated by co-transformation of RAWM1 with pJY8P2, containing the fad\(_{42}^{G42R}\) allele, and pCN5, including the niaD allele.

![AF and CPA analysis by TLC](image)

Fig. 3. pkaA overexpression affects *ipnA* expression.
Total RNA was isolated from RKIS1 (wild type) and TKIS20.1 (*alcA(p)::pkaA*) cultures 0, 12, 24, and 48 h after transferring from MMG to MMG or from MMG to MMT (an *alcA*-inducing medium). The mycelia were pre-grown for 24 h before shift. A 1.1 kb fragment containing *ipnA* gene was used as a probe.

![pkaA overexpression affects *ipnA* expression](image)

Fig. 4. mRNA analysis of *ppo* genes in wild-type strains of *F. graminearum* and *F. sporotrichioides*.

![mRNA analysis of *ppo* genes in wild-type strains of *F. graminearum* and *F. sporotrichioides*](image)
not sporulation\(^6\). Because laeA expression is abolished in a pkaA overexpression background, it appears that LaeA is the first member in the G-protein signaling pathway where sporulation and secondary metabolite production are uncoupled. Loss of laeA reduces not only ST production but also PN production.

**Oxylipin-mediated signaling of sporulation and secondary metabolism**

**Background** Because spores are important for the initiation of an infection, there has been considerable focus on identifying factors regulating spore production in fungi. In contrast to the progress made in identifying intracellular factors regulating fungal sporulation such as G-protein signaling described above, little is known about the specific extracellular signals governing spore production. One of the few extracellular signals known to regulate both asexual and sexual spore development is unsaturated fatty acid, in particular linoleic acid and derivatives thereof.\(^7\) Linoleic acid has been shown to induce sporulation (either sexual or asexual) in *Alternaria tomato*,\(^10\) *Sclerotinia fructicola*,\(^11\) *Neurospora crassa*\(^12\) and *Aspergillus* spp.\(^7\) A mutant strain of *Aspergillus nidulans* that is unable to make linoleic acid and instead accumulates high levels of oleic acid is impaired in asexual spore production and produces higher levels of ascospores than wild type under certain conditions\(^13\). In *Neurospora crassa*, oleic acid is the predominant fatty acid found in developing asci and mature ascospores, whereas linoleic acid is the predominant fatty acid in asexual tissue in this fungus\(^14\). Recently, Trail and Common\(^15\) proposed that lipid accumulation in perithecial-forming strains of *F. graminearum* may be harnessed for ascospore production. Taken together, these reports suggest that fatty acid metabolism is important for normal spore development.

Detailed studies of *Aspergillus* spp. suggest that oxygenated spp. of unsaturated fatty acids,\(^7\)\(^-\)\(^9\) known as oxylipins, are specifically involved in sporulation. In 1987, Champe *et al.* reported the detection of a secreted substance, called psi factor (for precocious sexual inducer), that induced premature cleistothecia formation and sexual sporulation and blocked conidiation in *A. nidulans*. Extensive chemical studies of the psi factor resulted in the identification of linoleic and oleic acid derived oxylipins. We now know that psi factor is a mixture of hydroxylated oleic (18:1), linoleic (18:2) and linolenic acid (18:3) derivatives (termed psiA \(\alpha, \beta, \gamma\), psiB \(\alpha, \beta, \gamma\) and psiC \(\alpha, \beta, \gamma\)\(^7\)\(^-\)\(^9\)) likely produced by all filamentous fungi.

Most recently, we have been able to clone three genes (e.g. *ppoA*, *ppoB* and *ppoC* for psi producing oxygenase) encoding dioxygeaneses that are likely to be responsible for psi production in *Aspergillus nidulans*\(^16\). The amino acid sequence of the encoding proteins shows very high similarity to that of the psi producing protein Lds from the fungus *Gaeumannomyces graminis*\(^17\). A putative ortholog has also been described in *Ustilago maydis* where it is found to be expressed in teliospores\(^18\). We have also found...
These three genes in *Fusarium* species including *F. graminearum*. As described below, these genes are not only important for sporulation but also secondary metabolism in both *Aspergillus* and *Fusarium* species.

**Deletion of ppo genes impacts sporulation and mycotoxin production in Aspergillus and Fusarium** An examination of a *ppoA* deletion strain of *A. nidulans* shows it to be defective in both spore and sterigmatocystin production. Deletion of *ppoA* significantly reduced the level of psiB1 and increased the ratio of asexual to sexual spore numbers four-fold. In contrast, forced expression of *ppoA* resulted in elevated levels of psiB1 and decreased the ratio of asexual to sexual spore numbers six-fold. Additionally, the *ppoA* deletion strain showed aberrant ST synthesis.

To determine if *ppo* genes could affect development in a similar fashion in *Fusarium*, we attempted to identify these genes in three *Fusarium* species. Sequence data from a *F. verticillioides* EST (expressed sequence tag) indicated it to be a likely *ppo* gene (e-value ca. -75 to *A. nidulans* *ppoA*). We amplified this DNA sequence (which we call *Fvppo1*) from *F. verticillioides* genomic DNA, sequenced *Fvppo1* to confirm identity, and then used *Fvppo1* to probe *F. verticillioides*, *F. graminearum* and *F. sporotrichioides* cosmid libraries. Each *Fusarium* species contained several strongly hybridizing cosmids. Subcloning and sequencing of these cosmids yielded putative *ppo* genes. Subsequent to this, BLAST analysis of the newly released *F. graminearum* genome (http://www-genome.wi.mit.edu/annotation/fungi/) revealed the presence of all three *ppo* genes called *Fgppo1*, *Fgppo2* and *Fgppo3* (Table 1). Transcript analysis of these three genes is shown in Fig. 4.

A *Fvppo1* disruption vector was created in which the hygromycin resistance gene, *hygB*, was ligated between *Fvppo1* flanking DNA (e.g. ca. 1 kb of flank 5’ and 3’ to the *Fvppo1* ORF). This vector was then used to transform *F. sporotrichioides* to hygromycin resistance. Two *ppo* deletion mutants were obtained. Examination of these Δ*ppo* strains of *F. sporotrichioides* indicate that T-2 toxin gene expression is greatly reduced (Fig. 5).

**Fig. 5.** mRNA analysis of *ppo1* and *tri6* in wild-type and Δ*ppo1* strains of *F. sporotrichioides.*

Note that deletion of *ppo1* results in a great decrease in *tri6* expression. *Tri6* encodes a regulatory gene required for the production of the trichothecene T2 toxin in *F. sporotrichioides.*
Table 1. Homology of Fusarium dioxygenases to linoleate diol synthase (lds) gene of Gaeumannomyces graminis

<table>
<thead>
<tr>
<th></th>
<th>E value</th>
<th>% identity</th>
<th>% similarity</th>
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<tbody>
<tr>
<td><em>F. graminearum</em></td>
<td>Ppo1</td>
<td>0.0</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Ppo2</td>
<td>1X10^{-98}</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Ppo3</td>
<td>1X10^{-158}</td>
<td>40</td>
</tr>
<tr>
<td><em>F. verticilloides</em></td>
<td>Ppo1</td>
<td>1X10^{-158}</td>
<td>39</td>
</tr>
<tr>
<td><em>F. sporotrichoides</em></td>
<td>Ppo1</td>
<td>1X10^{-157}</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2. Spore production and oxylipin content of wild type and Δppo1 strains of *F. sporotrichoides*.

<table>
<thead>
<tr>
<th></th>
<th>Conidia/μl 1</th>
<th>8-HOE 2</th>
<th>8-HODE</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>6.6X10^4</td>
<td>8.3</td>
<td>41.0</td>
</tr>
<tr>
<td>Δppo1</td>
<td>3.2X10^4</td>
<td>1.9</td>
<td>1.9</td>
</tr>
</tbody>
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1 The number of spores are statistically different between wild type and Δppo1 with Δppo1 consistently producing ca. 1/2 the amount of spores as wild type.
2 8-HOE = 8 hydroxyoleic acid or psiβ. 8-HODE = 8-hydroxylinoleic acid or psiα. In mg/g mycelium (dry weight).

and T-2 toxin production has been inhibited (Plattner, Devi and Keller, data not shown). In addition, asexual spore production is severely reduced compared to that of wild type and oxylipin content altered (Table 2).

Taken together, these data suggest that, similar to the G-protein signaling, oxylipin signaling affects both sporulation and secondary metabolism. Unlike members of the G-protein cascade, the *ppo* genes appear unique to fungi (Tsitsigiannis and Keller, data not shown). We speculate that oxylipins generated from the *ppo* gene products may act as ligands initiating several signal transduction cascades governing global developmental pathways.

Conclusion

The two pathways described in this paper, one signaling internally (G-protein) and one externally (oxylipin), highlight the intimate connection of secondary metabolite production and sporulation. Although both pathways are also tightly associated with aspects of primary metabolism and have far-reaching effects on colony development, the linkage of sporulation and metabolite production is not spurious or even indirect. For example a *brlA* binding site in the promoter of *aflR* suggests that this sporulation specific
transcription factor may be involved in ST regulation. The reasons for this co-regulation of sporulation and metabolite production are most likely to be discovered at the ecological and organismal level. It is tempting to speculate that the simultaneous regulation of both processes is associated with protective properties (e.g. allelopathic or anti-herbivoric chemicals, UV damage mitigation) of secondary metabolites in a sporulating colony.

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