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Review

Signalling pathways connecting mycotoxin production and sporulation

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SUMMARY

Mycotoxin contamination of food and feed presents a serious food safety issue on a global scale, causing tremendous yield and economic losses. These toxins, produced largely by members of the genera *Aspergillus* and *Fusarium*, represent a subset of the impressive array of secondary metabolites produced by filamentous fungi. Some secondary metabolites are associated temporally and functionally with sporulation. In *Aspergillus* and *Fusarium*, sporulation and mycotoxin production are both regulated by G protein signalling pathways. G protein signalling pathways commonly regulate fungal development, stress response and expression of virulence traits. In addition, fungal development is influenced by external factors. Among these are lipids, and in particular, oxylipin signals, which may be derived from either the fungus or infected seeds. Regardless of origin, oxylipins have the potential to elicit profound changes in both sporulation and mycotoxin production in the fungus. Signal transduction via G protein signalling pathways represents one mechanism by which oxylipin signals might elicit these changes. Therefore, in this review we integrate discussion of oxylipin signals and of G protein signalling cascades as regulators of fungal development.

INTRODUCTION

Fungi are prodigious producers of secondary metabolites, some with great significance to society as antibiotics or toxins. Mycotoxins can be defined as small organic molecules produced by filamentous fungi that 'evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route' (Bennett, 1987). The oldest recognized example of mycotoxin poisoning is ergotism, caused by ingestion of grain contaminated with ergot alkaloids from the phytopathogen *Claviceps purpurea* (e.g. reviewed by Bennett and Klich, 2003). Mycotoxin contamination of food and feed presents a serious food safety issue on a global scale; therefore, agricultural commodities traded nationally and internationally are screened and regulated for certain mycotoxins. Although it is impossible to determine the actual economic losses due to mycotoxin contamination of crops, a recent estimate puts annual losses at between $418 million and $1.66 billion to agribusiness in the United States (Robens and Cardwell, 2005; Vardon *et al.*, 2003). Among the most economically damaging mycotoxins are aflatoxins, produced by *Aspergillus flavus*, *Aspergillus parasiticus* and related species (e.g. Goto *et al.*, 1996); tricothecenes, including deoxynivalenol (also known as vomitoxin) and T-2 toxin, produced primarily by *Fusarium* species (Miller, 2002); fumonisins, produced primarily by *Fusarium* spp. or *Alternaria* (Miller, 2002); zearalenone produced by *Fusarium* spp. (Miller, 2002); and ochratoxin produced by *Aspergillus* and *Penicillium* spp. (Niessen *et al.*, 2005). The function of the vast majority of these metabolites for the producing organism remains enigmatic. However, intensive study of these toxins has yielded significant information regarding the regulation of their production. Many excellent reviews exist that address the biochemistry, physiology and genetics (e.g. Keller *et al.*, 2005; Payne and Brown, 1998; Sweeney and Dobson, 1998; Yu, 2004), molecular regulation (e.g. Calvo *et al.*, 2002; Sweeney and Dobson, 1999; Yu and Keller, 2005), and host genetics and cultural practices (e.g. Brown *et al.*, 2004; Munkvold, 2003) affecting mycotoxin production. Here we will focus on signalling pathways connecting sporulation with mycotoxin production, and discuss advances in our understanding of the role of lipids in these processes.

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SIGNAL TRANSDUCTION

Pathway-specific regulation of mycotoxin production

One remarkable property of secondary metabolites produced by fungi (including phytotoxins, antibiotics, pharmaceuticals and mycotoxins) is that the genes involved in their biosynthesis and

regulation are frequently clustered, much as they are in prokaryotes (reviewed in Keller and Hohn, 1997; Zhang *et al*., 2004). This clustering has been demonstrated for the biosynthetic pathways of the ergot alkaloids of *C. purpurea* (Tudzynski *et al*., 1999), ochratoxin from *Penicillium nordicum* (Karolewiez & Geisen, 2005), tricothecenes, zearalenone and fumonisins in *Fusarium* spp. (e.g. Hohn *et al*., 1993; Kim *et al*., 2005; Proctor *et al*., 2003), and aflatoxin/sterigmatocystin production in the Aspergilli (reviewed in Yu *et al*., 2004b; Zhang *et al*., 2004). The genome sequence of *Aspergillus nidulans* and three other Aspergilli, *A. flavus*, *A. orzyae* and *A. fumigatus* (Galagan *et al*., 2005; Machida *et al*., 2005; Nierman *et al*., 2005) revealed the potential existence of approximately 40 secondary metabolite gene clusters in each one of these species (Keller *et al*., 2005). Most identified clusters encode unknown metabolites, but the aflatoxin (*A. flavus/A. oryzae*) and sterigmatocystin (*A. nidulans*) gene clusters are exceptionally well studied (Keller *et al*., 2005). These latter two will be described briefly as representing mycotoxin gene clusters.

The sterigmatocystin biosynthetic gene cluster contains 25 co-regulated transcripts, encoding at least 21 enzymatic steps, within a 60-kb region of chromosome IV of *A. nidulans* (Brown *et al*., 1996; reviewed in Yu *et al*., 2004b). Sterigmatocystin is the penultimate precursor for aflatoxin. Reflecting this, the sterigmatocystin gene cluster in *A. nidulans* and the ~75-kb aflatoxin gene clusters of *A. flavus* and *A. parasiticus* contain homologous genes encoding the same enzymatic functions, although gene order has not been conserved (Yu *et al*., 2004a; reviewed in Yu *et al*., 2004b). By contrast, the gene clusters of *A. flavus* and *A. parasiticus* that encode aflatoxin biosynthesis are syntenic, and the individual genes share > 95% nucleotide homology between species (Ehrlich *et al*., 2005; Yu *et al*., 1995). In strains of *A. oryzae*, a species used by the food industry, the aflatoxin biosynthetic gene cluster can be found both in its entirety and fragmented (reviewed in Zhang *et al*., 2004).

The sterigmatocystin/aflatoxin biosynthetic gene clusters include genes encoding two different transcriptional regulators: *aflR* (Chang *et al*., 1993; Payne *et al*., 1993; Woloshuk *et al*., 1994; Yu *et al*., 1996a) and *aflJ* (Chang, 2003; Meyers *et al*., 1998). AflR encodes a $Zn(II)_2Cys_6$ domain protein (Woloshuk *et al*., 1994), which binds the palindromic sequence 5′-TCG(N5)GCA-3′ found in promoters of aflatoxin and sterigmatocystin biosynthetic genes (Ehrlich *et al*., 1999; Fernandes *et al*., 1998). In *A. flavus* and *A. parasiticus*, AflR also binds the sequence 5′-TTAGGCCTAA-3′ (Chang *et al*., 1995, 1999; Ehrlich *et al*., 1998) in its own promoter and those of aflatoxin genes, up-regulating gene expression. Studies in *A. parasiticus* suggest that AflR is necessary, but not sufficient, for aflatoxin gene expression (Kale *et al*., 2003). Transcribed divergently from *aflR* in the sterigmatocystin/aflatoxin gene cluster is *aflJ*. Deletion of *aflJ* reduces aflatoxin and sterigmatocystin production and biosynthetic gene transcription via an as yet unknown mechanism (R.A. Butchko & N.P. Keller, unpublished data; Meyers *et al*., 1998). One study (Chang, 2003) provides evidence for a physical interaction between AflJ and AflR.

The significance of biosynthetic gene clusters in filamentous fungi has long been debated. One hypothesis is that they represent an extended form of selfish genes, facilitating simultaneous mobilization of a discrete biosynthetic function for horizontal transfer (Walton, 2000). Another rationalization is that clustering is associated with co-regulation of genes within the cluster, reminiscent of operons and regulons in prokaryotes (reviewed in Zhang *et al*., 2004). However, eukaryotes effectively orchestrate the functioning of many biosynthetic pathways for which the various genes are dispersed throughout the genome. Recently, a novel protein was described that lends new credence to the regulation-by-clustering hypothesis. The nuclear protein LaeA is a predicted protein methyltransferase that acts as a global regulator of secondary metabolite gene expression in *Aspergillus* spp. (Bok and Keller, 2004; Bok *et al*., 2005). Genome-wide transcript analyses via microarray comparisons between wild-type *A. nidulans* and a Δ*laeA* strain showed transcriptional differences that were precisely demarcated by the predicted or known borders of secondary metabolite gene clusters (Bok *et al*., 2006). The aflatoxin and sterigmatocystin pathways are among the secondary metabolite gene clusters under the control of the global regulator, LaeA (Bok and Keller, 2004; Bok *et al*., 2006; N.P. Keller *et al*., unpublished results). Homologues of *laeA* also occur in several other genera of filamentous fungi. LaeA is notable in that, unlike other global regulators of secondary metabolism (e.g. the Cys_2His_2 zinc finger global transcription factors CreA, AreA and PacC that relay environmental signals; reviewed by Yu and Keller, 2005), it does not appear significantly to affect growth or levels of spore production. Secondary metabolism and sporulation are often linked in filamentous fungi, sharing some (but not all) regulatory elements (Adams and Yu, 1998; Calvo *et al*., 2002). Δ*laeA* strains represent an uncoupling of these events, allowing, for the first time, the study of global regulation of secondary metabolism in a background normal for growth and development.

Joint regulation of secondary metabolism and sporulation by G protein signalling

Filamentous fungi undergo distinct life-cycle phases of growth (accumulation of undifferentiated hyphae) and reproduction (elaboration of fruiting structures). Switching between these two phases is highly regulated (e.g. see Adams *et al*., 1998; Fischer, 2002 for reviews) and initiation is governed by perception of a combination of physiological and environmental conditions (reviewed in Calvo *et al*., 2002; Sweeney and Dobson, 1998). Some of these conditions, such as light (Mooney and Yager, 1990), nutrient source (Bennett *et al*., 1979), pH (Keller *et al*., 1997), metals (Cuero and Ouellet, 2005) and host plant chemistry

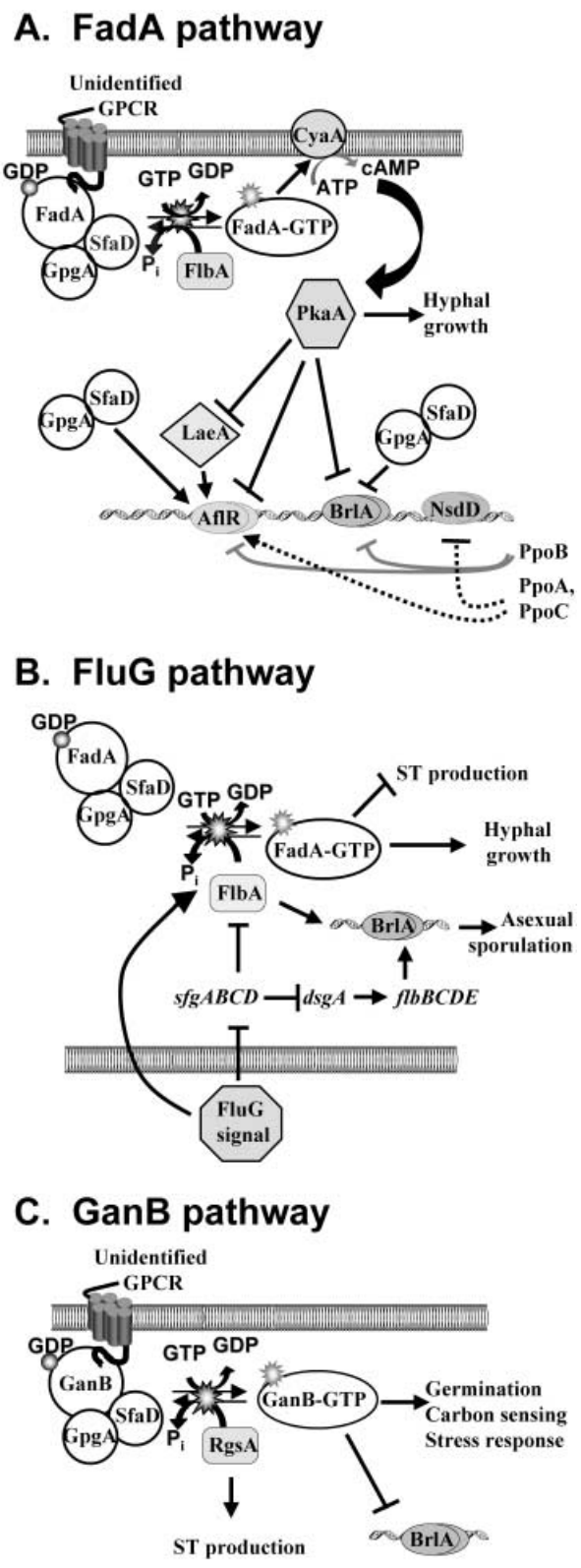
(e.g. Goodrich-Tanrikulu *et al.*, 1995; Zeringue, 2000), also affect mycotoxigenesis. Among the developmental changes during this phase switch are changes in secondary metabolite profiles. Fungal secondary metabolism and sporulation are associated both temporally and functionally (reviewed by Adams and Yu, 1998; Calvo *et al.*, 2002). Illustrating the latter, secondary metabolites in *Aspergillus* and *Fusarium* (including the mycotoxin zearalenone) are associated with the onset of sporulation (Calvo *et al.*, 2001; Champe and El-Zayat, 1989; Champe *et al.*, 1987; Mazur *et al.*, 1991; Wolf and Mirocha, 1973). Some secondary metabolites act as pigments protecting spores (Kawamura *et al.*, 1999; Ke and Luckner, 1979). Many secondary metabolites have antimicrobial activity (Peláez, 2005), suggesting roles as defence chemicals. Sterigmatocystin appears to be important for sporulation in *A. nidulans*: in a set of mutants incrementally blocked in sterigmatocystin biosynthesis at early, mid and late precursors, more conidia accumulated with each progressive conversion in the biosynthetic pathway, correlating conidiation to sterigmatocystin biosynthesis (Wilkinson *et al.*, 2004).

The processes of sporulation and secondary metabolite production have been demonstrated to share common regulatory elements. For instance, in *Colletotrichum lagenarium*, deletion of a mitogen-activated kinase (MAPK) gene lowers both production of conidia and expression of melanin genes (Takano *et al.*, 2000). In *Fusarium verticillioides*, a cyclin-like gene (*FCC1*) influences both asexual sporulation and production of the mycotoxin fumonisin B_1 (Shim and Woloshuk, 2001). G protein signalling pathways commonly regulate fungal development, stress response and expression of virulence traits, and their involvement in these processes has been shown for phytopathogens including *Cryphonectria parasitica*, *Magnaporthe grisea* and *Ustilago maydis*, as well as the model *Neurospora crassa* and the opportunistic human pathogen *Cryptococcus neoformans* (reviewed in Lee *et al.*, 2003; Lengeler *et al.*, 2000). In the Aspergilli, one of the first genetic insights linking sporulation with mycotoxin production was that both are regulated by members of a G protein signalling pathway (Hicks *et al.*, 1997; McDonald *et al.*, 2004). Intensive subsequent genetic and biochemical exploration of this pathway has begun to reveal the signalling circuitry connecting aflatoxin/sterigmatocystin production and sporulation in *Aspergillus*, which we will discuss in detail below.

The binding of a ligand to a G protein-coupled receptor (GPCR) is the event that activates G protein signalling. GPCRs reside in the cell membrane, where they perceive extracellular signals such as light, ions, amino acids, sugars, nucleic acids, steroids, polypeptides and fatty acids, and transduce this information about the external environment across the membrane to heterotrimeric G proteins comprising Gα, Gβ and Gγ subunits (e.g. reviewed by Pierce *et al.*, 2002). Briefly, these first steps in transmembrane signal transduction can be broken down thus: GPCR activation by ligand binding catalyses guanine nucleotide exchange (GTP for GDP) at the Gα subunit of an associated heterotrimeric G protein. Gα-GTP dissociates from Gβγ, and remains so until the intrinsic Gα GTPase hydrolyses the bound GTP to GDP again (e.g. reviewed by Dohlman, 2002; Hoffman, 2005). While dissociated, Gα and/or the Gβγ complex relay messages to other downstream effectors; in fungi these second messenger pathways are primarily (i) MAP kinase protein phosphorylation cascades and (ii) adenylyl cyclase/cAMP/PKA pathways (reviewed by Lengeler *et al.*, 2000). The signal is quenched when Gαβγ reassociates. GTP hydrolysis by Gα can be enhanced by RGS (regulators of G-protein signalling) proteins, expediting deactivation of G protein signalling and providing fine tuning for the system (see Siderovski *et al.*, 1999). As we currently understand it, the G protein signalling cascade linking sporulation and secondary metabolism in *Aspergillus* is centred on a G protein/cAMP/PKA signal transduction pathway. cAMP levels depend on the opposing activities of adenylyl cyclase and phosphodiesterases. In the G protein/cAMP/PKA pathway, adenylyl cyclase is stimulated (or repressed) by either Gα or Gβγ, resulting in increased or decreased accumulation of the second messenger, cyclic AMP (cAMP). cAMP is known to activate protein kinase A (PKA) (e.g. reviewed in Neves *et al.*, 2002; Pawson and Scott, 2005; Pierce *et al.*, 2002). In its inactive form, PKA comprises a holoenzyme of two regulatory and two catalytic subunits, which dissociate when cAMP binds the regulatory subunits. The freed catalytic subunits then perpetuate the signalling cascade by phosphorylating multiple cellular substrates. G protein/cAMP/PKA signalling is known to govern morphogenesis of a number of plant pathogens, including *U. maydis*, *M. grisea*, *Cryphonectria parasitica*, *Colletotrichum* spp. and *Fusarium* spp. (for a review see Lee *et al.*, 2003). However, signals and signal perception via GPCRs associated with these pathways are largely unknown. In the following sections, we focus on current knowledge regarding this pathway and its relationship to mycotoxigenesis in *Aspergillus* and *Fusarium* (see Fig. 1 and Table 1 for an overview).

Heterotrimeric G protein subunits

Filamentous fungi commonly possess three heterotrimeric G protein Gα subunits (Bölker, 1998). In *A. nidulans*, the Gα subunits are represented by FadA (Yu *et al.*, 1996b), GanA (Chang *et al.*, 2004; Han *et al.*, 2004b) and GanB (Chang *et al.*, 2004). Currently, only single Gβ (*sfaD*) and Gγ (*gpgA*) alleles have been identified. A dominant activating mutation in *fadA* (i.e. the $fadA^{G42R}$ allele) resulted in a loss of sterigmatocystin biosynthesis and asexual sporulation (Hicks *et al.*, 1997; Shimizu and Keller, 2001; Yu *et al.*, 1996b), indicating that one role of FadA in development is to suppress these processes. The *flbA* gene encodes a protein bearing a regulator of the G protein signalling (RGS) domain. Loss of function mutations in *flbA* resemble dominant activating mutations in *fadA* (Hicks *et al.*, 1997; Lee and Adams, 1994b; Yu *et al.*, 1996b), confirming that at least one role of FlbA is the



inactivation of FadA-GTP. Negative effects on sterigmatocystin production and conidiation following loss of FlbA or activation of FadA function are mediated partially through changes in the expression of genes for the transcriptional regulators of these processes: *aflR* and *brlA*, respectively (Hicks *et al.*, 1997; Lee and Adams, 1996). FlbA also appears to exert post-transcriptional repression on AflR (J. Hicks and N.P. Keller, unpublished data; Shimizu *et al.*, 2003); this repression does not appear to be mediated by FadA, and is independent of that ascribed to PkaA (detailed below).

FlbA also appears to be under regulation by additional proteins. Mutations in *flbA* were suppressed by second-site mutations in at least four other loci, designated *sfaA*, *sfaC*, *sfaD* and *sfaE* (Yu *et al.*, 1999). One of these, *sfaD*, was found to encode the heterotrimeric G protein β subunit for *A. nidulans*. A recent study (Seo and Yu, 2006) suggests that SfaD activity requires the phosducin-like protein PhnA. SfaD and PhnA contribute positively to vegetative growth signalling and sexual development, while repressing asexual development. The third member of the heterotrimer is GpgA (Gγ). When partnered with GanB-GTP, SfaD::GpgA appears to regulate carbon sensing, germination and stress response (Lafon *et al.*, 2005; Seo *et al.*, 2005). When partnered with FadA-GTP, SfaD::GpgA is part of the pathway inducing normal growth and sexual sporulation (Rosén *et al.*, 1999; Seo *et al.*, 2005). The FadA-GTP/SfaD::GpgA pathway opposes sterigmatocystin production, partially through PkaA inhibition of *aflR* gene expression. However, SfaD regulation of *alfR* expression opposes that of PkaA: deletion of either PhnA or SfaD diminished transcription of *aflR* in *A. nidulans* and over-expression of *aflR* restored sterigmatocystin production to an *sfaD* mutant (Seo and Yu, 2006).

Epistasis analyses also suggest activation of FlbA (and subsequent FadA repression) by the product of the *fluG* gene. Although

Fig. 1 Models of regulatory pathways for sporulation and secondary metabolite production in *A. nidulans*, as linked by components of G protein signalling pathway(s). Arrowheads indicate positive influences; blocked lines indicate negative effects. Depicted are: Gα (FadA and GanB), Gβ (SfaD) and Gγ (GpgA) heterotrimeric G protein subunits, adenylate cyclase (CyaA), an FadA-regulating RGS protein (FlbA), a GanB-regulating RGS protein (RgsA), protein kinase A (PkaA) and an unidentified G protein-coupled receptor (GPCR). Also shown are psi-producing oxygenases (PpoA, PpoB and PpoC) and a global regulator of secondary metabolite gene clusters (LaeA). BrlA and NsdD are transcriptional regulators that are required for asexual and sexual sporulation, respectively. The transcriptional regulator AflR activates expression of sterigmatocystin (ST) biosynthetic genes. FluG produces a diffusible signal that remains to be chemically characterized. Loci designated *sfg* represent suppressors of (mutation in) *fluG*; *dsgA* is a dominant suppressor of (mutation in) *fluG*; loci designated *flb* are named for the mutant phenotype of 'fluffy, low *brlA* expression' (reviewed in Adams *et al.*, 1998). Direct interactions between components have not been experimentally tested. (A) FadA pathway. (B) FluG pathway. (C) GanB pathway.

Table 1 Effects of mutations in G protein signalling pathway genes on spore and mycotoxin production.

Gene	Fungal species	Predicted protein	Mutant genotype	Effect of mutation on sporulation	Effect of mutation on mycotoxin production	Reference
gprA	*A. nidulans*	GPCR	Δ*gprA*	inhibited homothallic cleistothecia formation, but not asexual sporulation	not reported	Seo *et al.* (2004)
gprB	*A. nidulans*	GPCR	Δ*gprB*	inhibited homothallic cleistothecia formation, but not asexual sporulation	not reported	Seo *et al.* (2004)
gprD	*A. nidulans*	GPCR	Δ*gprD*	enhanced cleistothecia production; repressed asexual sporulation	not reported	Han *et al.* (2004a)
fadA	*A. nidulans*	heterotrimeric G protein α subunit	Δ*fadA*	no change in conidiation; blockage of cleistothecial production	reduced ST* gene transcription	Hicks *et al.* (1997); Rosén *et al.* (1999)
	A. nidulans		$fadA^{G42R}$†	reduced conidiation	reduced ST gene transcription	Hicks *et al.* (1997)
	A. nidulans		$fadA^{G203R}$‡	enhanced conidiation; blockage of cleistothecial production	enhanced ST gene transcription	Hicks *et al.* (1997); Rosén *et al.* (1999)
	F. sporotrichioides		$fadA^{G42R}$	decreased conidiation	enhanced T2§ production	Tag *et al.* (2000)
	A. parasiticus		$fadA^{G42R}$	decreased conidiation	loss of NOR (aflatoxin precursor)	Hicks *et al.* (1997)
	A. flavus		$fadA^{G42R}$	decreased conidiation	repressed production of aflatoxin and cyclopiazonic acid	McDonald *et al.* (2004)
ganB	*A. nidulans*	heterotrimeric G protein α subunit	Δ*ganB*	decreased conidiation	no effect	Chang *et al.* (2004); Han *et al.* (2004b)
	A. nidulans		$ganB^{G207R}$¶	decreased conidiation	not reported	Chang *et al.* (2004)
	A. nidulans		$ganB^{Q208L}$**	decreased conidiation	not reported	Chang *et al.* (2004)
sfaD	*A. nidulans*	heterotrimeric G protein β subunit	Δ*sfaD*; point mutations	conidiation in liquid culture; blockage of cleistothecial production	loss of ST production and gene transcription	Rosén *et al.* (1999); Yu *et al.* (1999); Seo and Yu (2006)
gpgA	*A. nidulans*	heterotrimeric G protein γ subunit	Δ*gpgA*	delayed conidiation; inhibition of cleistothecium formation	loss of ST production and gene transcription	Seo *et al.* (2005); Seo and Yu (2006)
pkaA	*A. nidulans*	cAMP-dependent PKA	Δ*pkaA*	enhanced conidiation	delayed ST gene transcription; restored ST gene transcription to Δ*flbA* mutants	Shimizu and Keller (2001)
	A. nidulans		OE::*pkaA*	reduced conidiation	loss of ST gene transcription	Shimizu and Keller (2001)
flbA	*A. nidulans*	RGS‡‡ domain-bearing protein; deactivation of FadA	Δ*flbA*	reduced conidiation	reduced ST gene transcription	Hicks *et al.* (1997); Lee and Adams (1994b, 1996)
	A. nidulans		OE::*flbA*††	enhanced conidiation	enhanced ST gene transcription	Hicks *et al.* (1997); Lee and Adams (1994b, 1996)
rgsA	*A. nidulans*	RGS domain-bearing protein; deactivation of GanB	Δ*rgsA*	failed to restore conidiation to Δ*flbA* mutant	reduced ST production	Han *et al.* (2004b)
	A. nidulans		OE::*rgsA*	conidiation in liquid culture§§	not reported	Han *et al.* (2004b)
fluG	*A. nidulans*	Activation of FlbA	Δ*fluG*	reduced conidiation	reduced ST gene transcription	Hicks *et al.* (1997); Lee and Adams (1994b, 1996)
	A. nidulans		OE::*fluG*††	enhanced conidiation	no effect on ST gene transcription	Lee and Adams (1994b, 1996); Hicks *et al.* (1997)
sfaA	*A. nidulans*	unknown	point mutation	restored conidiation to *flbA* mutants	returned ST production to *flbA* mutants	Yu *et al.* (1999)
sfaC	*A. nidulans*	unknown	point mutation	restored conidiation to *flbA* mutants	returned ST production to *flbA* mutants	Yu *et al.* (1999)
sfaE	*A. nidulans*	unknown	point mutation	restored conidiation to *flbA* mutants	returned ST production to *flbA* mutants	Yu *et al.* (1999)

*ST = sterigmatocystin.
†$fadA^{G42R}$, mutation predicted to eliminate the intrinsic GTPase activity of the Gα subunit, thereby preserving its active state.
‡$fadA^{G203R}$, mutation preserving FadA in an inactive state.
§T2 = trichothecene mycotoxin T2.
¶$ganB^{G207R}$, mutation preserving GanB in an inactive state.
**$ganB^{Q208L}$, mutation preserving GanB in a constitutively active state.
††OE::*flbA*; OE::*fluG*: genes transcriptionally fused to the threonine-inducible alcA promoter.
‡‡RGS = Regulator of G protein signalling.
§§Wild-type *A. nidulans* does not normally form conidia when submerged in liquid culture.

the function of the predicted 96-kDa, cytoplasmically localized FluG protein remains enigmatic, one clue is that only the C-terminal portion of FluG (which is similar to glutamine synthetase I) is necessary for inducing conidiation in *A. nidulans*, via production of a small, diffusible, extracellular signal (D'Souza *et al.*, 2001; Lee and Adams, 1994a,b, 1996). FluG exerts a positive effect on sterigmatocystin production that appears to be mediated by FlbA (Hicks *et al.*, 1997; Lee and Adams, 1994b, 1996). It also triggers a separate regulatory pathway that culminates in asexual reproduction (Fig. 1b). Common to both pathways are *sfg* loci (for *s*uppressors of Δ*fluG*) whose mutation resulted in suppression of Δ*fluG* (Seo *et al.*, 2003). Separate from the FlbA pathway is *dsgA*, which is predicted to encode a positive regulator mediating the effects of FluG or FlbA on conidiation, but not on sterigmatocystin production (D'Souza *et al.*, 2001). Also independent of the FlbA pathway but regulating *brlA* expression are at least four other loci (*flbB, flbC, flbD* and *flbE*) (Adams *et al.*, 1998; Wieser *et al.*, 1994). This asexual development pathway competes with the FadA-GTP/SfaD::GpgA growth pathway; mutations in *fadA* (Gα), *sfaD* (Gβ) or *gpgA* (Gγ) all overcome the fluffy-autolytic phenotype of an *flbA* deletion mutant, restoring conidiation (Seo *et al.*, 2005; Yu *et al.*, 1996b, 1999).

In addition to FadA, *A. nidulans* is known to bear at least two other Gα proteins, with their own signalling pathways. Currently, little is known about GanA function. GanB is implicated in nutrient or stress sensing, and positively regulates conidial germination in response to carbon sensing, via cAMP/PKA signalling (Chang *et al.*, 2004; Han *et al.*, 2004b; Lafon *et al.*, 2005). In an otherwise wild-type background, deleting or inactivating *ganB* reduced condial production by half or more on solid medium. However, over-expression or constitutive activation of *ganB* did not have the opposite effect; rather, these mutants were almost entirely devoid of conidia (Chang *et al.*, 2004). Like FadA, GanB is deactivated by an RGS domain protein. This protein, RgsA, is functionally unique from FlbA, the RGS domain protein described above to deactivate FadA. Additionally, epistatic analyses support a specific interaction between RgsA and GanB, but not with FadA or GanA (Han *et al.*, 2004b), indicating that these cognate Gα–RGS pairings are specific and exclusive. Deletion of *ganB* had no obvious effect on sterigmatocystin production, but deletion of *rgsA* greatly reduced sterigmatocystin while allowing accumulation of other, unidentified pigments (Han *et al.*, 2004b). A Δ*rgsA*, Δ*ganB* double mutant was restored to wild-type levels of sterigmatocystin production, suggesting that RgsA/GanB interactions are required for normal sterigmatocystin production.

G protein subunits have also been shown to link regulation of secondary metabolism and sporulation in other fungi. Heterologous expression of the *A. nidulans fadA*^{G42R} dominant active allele in *Fusarium sporotrichioides* altered the timing of trichothecene gene expression and ultimately enhanced production of the trichothecene T2. Expression of *fadA*^{G42R} in *F. sporotrichioides* also reduced production of conidia (Tag *et al.*, 2000). This same allele, when expressed in *A. flavus*, repressed both aflatoxin biosynthesis and cyclopiazonic acid production (McDonald *et al.*, 2004). Expression of *fadA*^{G42R} in a strain of *A. parasiticus* whose aflatoxin biosynthetic pathway is blocked after synthesis of a precursor, norsolorinic acid, resulted in an aconidial strain unable to produce norsolorinic acid (Hicks *et al.*, 1997). In the chestnut blight-causing fungus *Cryphonectria parasitica, cpg1* mutants (defective in the Gα subunit) show decreased pigmentation and spore production (Gao and Nuss, 1996).

PKA and cAMP

Because PKA is a potential downstream constituent of Gα-GTP signalling, it was examined as a link in the signalling cascade that mediates FadA repression of *aflR* and *brlA* expression. *A. nidulans*, like other fungi (e.g. *Saccharomyces cerevisiae*, *U. maydis* and *M. grisea*; Lee *et al.*, 2003), bears two isoforms of the PKA catalytic subunit (PkaA and PkaB). Simultaneous deletion of both genes is lethal (Ni *et al.*, 2005). Deletion of *pkaB* does not cause obvious phenotypic differences, indicating that *pkaA* plays a more dominant role in growth and development. However, over-expression of *pkaB* can alleviate certain Δ*pkaA* phenotypes, including vegetative growth defects and delayed conidiospore germination (Ni *et al.*, 2005). Deleting the gene for the PkaA catalytic subunit of *A. nidulans* restored sterigmatocystin gene expression to a Δ*flbA* mutant and partially restored conidiation to Δ*flbA* and *fadA*^{G42R} (constitutively active FadA) mutants in *A. nidulans* (Shimizu and Keller, 2001). Over-expression of *pkaA* stifled expression of *brlA*, *aflR* and *stcU* (a sterigmatocystin biosynthetic gene) and reduced conidiation, although not to the levels seen in a *fadA*^{G42R} mutant. PkaA represses sterigmatocystin biosynthetic gene expression via negative transcriptional regulation of *aflR* gene expression, and also by post-transcriptional regulation (phosphorylation) of the AflR protein (Shimizu and Keller, 2001; Shimizu *et al.*, 2003). A later study showed that, in addition to *aflR*, PkaA negatively regulates expression of *laeA*, a global regulator of multiple *Aspergillus* secondary metabolite gene clusters (Bok and Keller, 2004).

RasA is a member of the family of small GTP-binding proteins, and plays a role in conidial germination for *A. nidulans* (Fillinger *et al.*, 2002; Osherov and May, 2000; Som and Kolaparthi, 1994). Shimizu *et al.* (2003) demonstrated its role in linking PkaA and FlbA to regulation of *aflR* expression. RasA, like PkaA, represses *aflR* transcriptionally and post-transcriptionally. Genetic studies showed that PkaA contributes to RasA post-transcriptional (but not transcriptional) regulation of AflR; however, RasA modulation of AflR activity does not involve phosphorylation (Shimizu *et al.*, 2003).

Like FadA, cAMP-dependent PKA appears to be a conserved factor in regulation of aflatoxin/sterigmatocystin production in Aspergilli. Biochemical feeding studies coupled with PKA activity assays suggested that an FadA/cAMP/PKA regulatory cascade

controls aflatoxin regulation in *A. parasiticus* (Roze *et al.*, 2004). These studies clarified that, in Aspergilli, FadA-mediated signalling is (at least in part) transmitted via a cAMP/PKA signalling cascade, and that PkaA plays a major role in activation of vegetative growth, and repression of both conidiation and aflatoxin/sterigmatocystin production.

GPCRs

Ligand perception by GPCR(s) represents the logical apex of this G protein signalling cascade, which governs both sporulation and secondary metabolism in Aspergilli. Ten genes (*gprA–gprK* but excluding a *gprJ* gene) predicted to encode GPCRs are found in the *A. nidulans* genome. Recent reports demonstrate that mutations in the GPCR-encoding genes *gprA*, *gprB* and *gprD* affect sexual development in *A. nidulans*. Specifically, *gprA* and *gprB* mutations disrupted sexual development in self-fertilization (Seo *et al.*, 2004). By contrast, a *gprD* mutation enhanced sexual development, but delayed conidial germination and slowed hyphal growth (Han *et al.*, 2004a). It seems likely that these GPCRs, like other components of heterotrimeric G protein signalling pathways, also might be involved in sterigmatocystin/aflatoxin production by *Aspergillus* spp.

LIPIDS

Shared intracellular signalling pathways for sporulation and mycotoxin production suggest a common trigger(s) for both these processes. In addition to the environmental factors mentioned above that govern these processes (nutrient source, pH, light, host plant chemistry), lipid signals also affect mycotoxigenesis and sporulation. Among lipid signals, of particular note are oxylipins, oxygenated fatty acid-derived molecules implicated as intra- and intercellular signals in animals, plants and fungi (Herman, 1998; Noverr *et al.*, 2003). As we describe in the paragraphs that follow, a series of genetic, biochemical and physiological studies in *Aspergillus* and *Fusarium* spp. strongly support a case for conserved, oxylipin-mediated signalling between fungus and host. Finally, oxylipins are logically tied to G protein signalling pathways in fungi. G protein signalling is initiated when an appropriate GPCR at the cell surface undergoes ligand perception. Oxylipin signalling and perception is best understood in mammalian systems, and in these cells oxylipin perception is achieved almost exclusively by GPCRs (Im, 2004; Metters, 1995; Tsuboi *et al.*, 2002).

Lipid pools available to mycotoxigenic fungi

Mycotoxigenic fungi are generally opportunistic pathogens of wounded or weakened hosts (e.g. Cotty *et al.*, 1994). During colonization, both *Aspergillus* and *Fusarium* species utilize hydrolytic enzymes which contribute to virulence and spread of the pathogen. Among these are pectinases, proteases, amylases, ligninases, lipases and others (Betts and Dart, 1989; Brown *et al.*, 1992, 2001; Chen *et al.*, 1999a,b,c; Cotty *et al.*, 1994; Shieh *et al.*, 1997). Lipases are important for *Aspergillus* and *Fusarium* pathogenesis. To access carbon sources from seeds and colonize the inner tissues, fungi must first penetrate the waxy, outer cuticular barrier. *Aspergillus* and *Fusarium* spp. both enter seeds via wounds, but *Fusarium* spp. are also capable of direct ingress through the cuticle. The major structural component of the cuticle is cutin, a polyester composed primarily of hydroxy and epoxy *n*-C16 and *n*-C18 fatty acids linked mainly by ester bonds (Carvalho *et al.*, 1999; Kolattukudy *et al.*, 1995). Enzymatic degradation of this polymer by lipase enzymes called cutinases is important in the invasion of plants by phytopathogenic fungi such as *Alternaria brassicicola* (Berto *et al.*, 1999), *Pyrenopeziza brassicae* (Li *et al.*, 2003), *Ascochyta rabiei*, *Botrytis cinearea*, *Erisyphe graminis* and others (reviewed by Carvalho *et al.*, 1999). The role of cutinase in fungal invasion has been especially well studied in *Fusarium solani* f. sp. *pisi* (Dickman *et al.*, 1989; Rogers *et al.*, 1994; Stahl and Schäfer, 1992). Cutinase activity serves the dual purpose of breaking through the cuticle and releasing energy-rich fatty acids from cutin polymers. Catabolism of other lipid pools, primarily triacylglycerides, for use as carbon sources in growth and development has been demonstrated as well. For example, lipids that are signals in *U. maydis* can also serve as carbon sources (Klose *et al.*, 2004). *Aspergillus* also has been shown to utilize lipids as growth substrates (e.g. Kawasaki *et al.*, 1995; Maggio-Hall and Keller, 2004; Mellon *et al.*, 2000, 2005). Preference for lipids over other available carbon sources may reflect the nutritional composition of the substrate. For example, Mellon *et al.* (2000) demonstrated that *A. flavus*, when grown on cotton-seed-simulating medium, utilized triglycerides only after exhaustion of sugars (Mellon *et al.*, 2000). By contrast, in corn-kernel-simulating medium, *A. flavus* simultaneously hydrolysed starch and triglycerides (Mellon *et al.*, 2002). In living corn kernels, *Aspergillus* was shown to target lipid bodies, rather than starch granules, for degradation during infection (Smart *et al.*, 1990). Tracking of substrate usage in *Aspergillus*-infected corn kernels (Mellon *et al.*, 2005) confirmed an initial drop in sugar (sucrose and raffinose) concentrations, followed by continuous triacylglyceride depletion after 2 days (concomitant with initial aflatoxin detection). Other studies have highlighted the importance of lipase to mycotoxigenic fungi. For example, in *Fusarium graminearum*, genetic studies have linked lipase production to virulence in wheat and maize (Voigt *et al.*, 2005). Yu *et al.* (2003) showed that a lipase gene, *lipA*, from *A. parasiticus* and *A. flavus* is induced by lipid substrate, and its expression generally correlates with aflatoxin production. These studies suggest the importance of lipid utilization to mycotoxigenic fungi. Below, we discuss effects of lipids on mycotoxin production through both metabolic and signalling routes.

Lipids as a carbon source fuelling mycotoxin production

In *Aspergillus*, fatty acids have long been associated with aflatoxin production. More than three decades ago, Jemmali and Guilbot (1974) reported that a saponified lipid fraction from wheat germ, containing mixed fatty acids, stimulated aflatoxin production by *A. flavus*. Production of aflatoxin by *Aspergillus parasiticus* and *Aspergillus flavus* is greater on lipid-rich than starchy seeds (Fabbri *et al*., 1980). Lipid precursors garnered from the host are thought to be stored in cytosolic lipid bodies, which commonly accumulate in *Aspergillus* spp. and other saprophytic fungi during growth, especially in resting or reproductive structures (Murphy, 2001). Fatty acids are then metabolized by β-oxidation pathways, which ultimately release acetyl-CoA moieties available for cell metabolism. The β-oxidation pathway has been partially examined in *A. nidulans*. In *A. nidulans*, as in mammals, β-oxidation occurs in both the peroxisomes and the mitochondria (Maggio-Hall and Keller, 2004). Long-chain (16C or more) fatty acid metabolism occurs in the peroxisomes. Examination of β-oxidation in *A. nidulans* and in the aflatoxin-producing species *A. flavus* and *A. parasiticus* strongly supported a case for β-oxidation-derived acetyl-CoA incorporation into sterigmatocystin and aflatoxin molecules (Maggio-Hall *et al*., 2005). The authors speculated that fungal β-oxidation, induced by seed fatty acids, directly contributes to the increased production of polyketides (which are secondary metabolites synthesized, like fatty acids, by condensation of short-chain carboxylic acids such as acetyl CoA). In addition to aflatoxin and sterigmatocystin, several other mycotoxins, including fumonisin, ochratoxin and zearalenone, are polyketide derivatives.

Lipids as signals

Lipids have been shown to regulate virulence and development, including both spore and mycotoxin production, in fungi. We discuss lipid influences on sporogenesis and mycotoxigenesis separately in the following two sections.

Morphogenesis and reproduction

Reproductive development of filamentous fungi has been shown to be influenced by perception of lipids (e.g. Calvo *et al*., 1999; Goodrich-Tanrikulu *et al*., 1998; Hyeon, 1976; Katayama and Marumo, 1978; Nukina *et al*., 1981; Rai *et al*., 1967). For *U. maydis*, the triacylglycerides comprising corn oil as well as individual or mixed fatty acids served not only as carbon sources, but also as signals to initiate filamentous growth needed for invading plant tissues. For example, as little as ~4 nM palmitic acid added to medium induced filamentous growth (Klose *et al*., 2004). Such a low concentration is negligible as a nutrient source, but is comparable with bioactive concentrations for other microbial signal molecules such as bacterial homoserine lactones (Eberhard *et al*., 1981; Eberl *et al*., 1996) or non-peptidyl fungal sex hormones such as antheridiol or trisporic acid (Gooday, 1983). A *U. maydis* PKA mutant was unable to respond to fatty acids and also exhibited less extracellular triacylglycerol lipase activity (Klose *et al*., 2004), suggesting that a lipid-triggered cAMP signalling pathway might contribute to the ability to metabolize lipids and autoinductively generate more lipid signals from plant substrates. In *Colletotrichum gloeosporioides*, cutin monomers derived from the surface waxes of avocado induced spore germination and appressorium formation (Kolattukudy *et al*., 1995; Podila *et al*., 1993). Similar to the observations described for *U. maydis* above, wax extract estimated at approximately 10 nM was sufficient to elicit a reponse (Podila *et al*., 1993) and appeared to involve a cAMP signalling pathway (Kim *et al*., 2001). However, lipid signalling in *Colletotrichum* appears to be more specific than that of *U. maydis* (Podila *et al*., 1993). Appressorium formation was most strongly initiated in response to long-chain fatty alcohols (C24 or longer); fatty acids and short-chain fatty alcohols had only a negligible stimulatory effect on appressorium formation. However, extracts from waxes of non-host plants, despite containing significant proportions of long-chain fatty alcohols, failed to induce (or even repressed) appressorium formation by *C. gloeosporioides* spores. Conversely, avocado wax was unable to stimulate appressorium formation by other *Colletotrichum* spp. (Podila *et al*., 1993). This study demonstrates that effects of individual lipids on spore development vary with fungal species. Linoleic acid in particular has a sporogenic effect on several genera including *Alternaria*, *Neurospora* and *Sclerotinia* (reviewed in Calvo *et al*., 2001). In *Aspergillus* spp., fatty acid stimulation of sporulation was dependent on chain length and presence of double bounds: in general, C18 unsaturated fatty acids had the greatest stimulatory effect (Calvo *et al*., 1999).

Mycotoxin production

Not only do lipid-rich tissues support fungal growth, but a correlation also has been observed between lipids and aflatoxin production (Fanelli and Fabbri, 1989; Fanelli *et al*., 1983, 1995). *Aspergillus* frequently infects oil-rich seeds (e.g. peanuts and tree nuts). Corn kernels, also notoriously susceptible to *Aspergillus* and aflatoxin contamination, have a lower overall oil content than nuts, but certain seed regions are oil-rich. Corn embryos contain approximately 9% starch, 31% lipid and 19% protein, in contrast to the endosperm which contains 88% starch, < 1% lipid and 7% protein (Earle *et al*., 1946). In corn, *A. flavus* and *A. parasiticus* predominantly colonized (and produced aflatoxin in) the lipid-rich embryo and aleurone tissues (Keller *et al*., 1994). The importance of seed oils to aflatoxin production by *Aspergillus* has also been shown in cotton seeds: extracting lipids from cottonseed meal reduced aflatoxin production by *A. flavus* approximately 1000-fold (Mellon *et al*., 2000). This lipid–mycotoxin association is not, however, universal. *F. verticilliodes*, which also

frequently infects maize kernels, colonizes the two major tissues of maize kernel, embryo and endosperm, equally well but produced five times more mycotoxin (fumonisin B1) in the endosperm (Shim *et al.*, 2003), which contains negligible lipid (Earle *et al.*, 1946).

Additional associations between lipids and mycotoxin production have been reported. In *Monascus ruber*, medium-chain fatty acids (C6 to C12) added to growth medium inhibited production of the mycotoxin citrinin (Hajjaj *et al.*, 2000). Free unsaturated fatty acids inhibited *Aspergillus* growth and aflatoxin production (Fanelli and Fabbri, 1989). In particular, epoxy fatty acids have been shown to stimulate aflatoxin production in *Aspergillus* (Fanelli and Fabbri, 1989; Fanelli *et al.*, 1983, 1995; Passi *et al.*, 1984). For example, linoleic acid, abundant in plant tissues, can readily become oxidized by reacting with free hydroxyl radicals, or enzymatically via the action of lipoxygenases and dioxygenases (Mueller, 2004). Fabbri *et al.* (1983) and Passi *et al.* (1984) demonstrated that peroxidized derivatives of linoleic acid enhanced aflatoxin production more than 100-fold over additions of unaltered linoleic acid, the effects of which were negligible. Indirect evidence correlated peroxy fatty acids with aflatoxin production as well: peroxidation of linoleic acid (enzymatic, via lipoxygenase, or non-enzymatic) and subsequent cleavage by hydroperoxide lyase generates hexanal, which appeared to inhibit fungal growth at low concentrations (Gardner *et al.*, 1996). Hexanal (along with linoleic acid) was a prominent constituent in maize genotypes that were repressive to growth and aflatoxin production by *A. flavus* (Zeringue *et al.*, 1996). These observations suggested that downstream products of plant lipoxygenases might have potential roles in aflatoxin regulation. Supporting this view, treatment of *Aspergillus* cultures with exogenous seed lipoxygenase products altered aflatoxin gene transcription (Burow *et al.*, 1997). In the following sections, we discuss the effects of plant lipoxygenase products (and the structurally similar products of fungal dioxygenases) on the production of aflatoxin/sterigmatocystin by *Aspergillus*.

Developmental effects of oxylipins on fungi

Oxylipins comprise a family of structurally related, oxygenated, long-chain fatty acid-derived molecules. Although oxygenated fatty acids can arise from non-enzymatic reactions in the cell, generation of certain species is catalysed by lipoxygenase enzymes in plants, and lipoxygenase or dioxygenase enzymes in fungi. Oxylipins serve as intra- and intercellular signals in animals, plants and fungi (Herman, 1998; Noverr *et al.*, 2003), mediating a number of functions ranging from regulation of reproduction in invertebrates to formation of innate defence mechanisms in plants (Howe and Schilmiller, 2002; Noverr *et al.*, 2003; Shah, 2005). Oxylipin production is ubiquitous among pathogenic and saprophytic fungi and appears to play a role in life-cycle control, particularly in sexual and asexual development (reviewed in Herman, 1998; Keller *et al.*, 2005; Noverr *et al.*, 2003; Yu and Keller, 2005). The broad occurrence of oxylipins, and structural similarity (Fig. 2) due to shared biosynthetic origins, suggests potential for cross-kingdom communication between closely associated organisms. In fact, hydroxylated C18 unsaturated fatty acids are naturally produced by *A. nidulans* (Champe and El-Zayat, 1989; Mazur *et al.*, 1990, 1991), and evidence exists for such communication in the *Aspergillus*/seed pathosystem.

Endogenous oxylipins affect sporulation and mycotoxin production

One of the first extracellular signals described to regulate both asexual and sexual spore development was psi (precocious sexual inducer) factor, the collective term for a series of oleic, linoleic and linolenic acid-derived oxylipins (Fig. 2) produced by *A. nidulans* (Calvo *et al.*, 2001; Champe and El-Zayat, 1989; Champe *et al.*, 1987; Mazur *et al.*, 1991). Alluding to hormone-like activity, at least two of the components (partially purified psiA1 and psiB1) had opposing effects on pigment production (Champe and El-Zayat, 1989). Moreover, one individual component (psiA1) demonstrated bioactivity at or above 0.2 μM—comparable with bioactive concentrations of trisporic acid, a fungal sex hormone of *Mucor* spp. (Champe and El-Zayat, 1989; Gooday, 1983). Similar oxylipins are also produced by other *Aspergillus* spp. (N.P. Keller and H.W. Gardner, unpublished data) and other fungal species (e.g. *Eremothecium sinecaudum*, *Laetisaria arvalis*, *Gaeumannomyces graminis*, *Fusarium oxysporum* and *Saccharomycopsis* spp.; Bareetseng *et al.*, 2004; Brodowsky and Oliw, 1993; Hamberg *et al.*, 1994; Nakayama *et al.*, 1996; Sebolai *et al.*, 2005; Su and Oliw, 1996; Su *et al.*, 1995). As

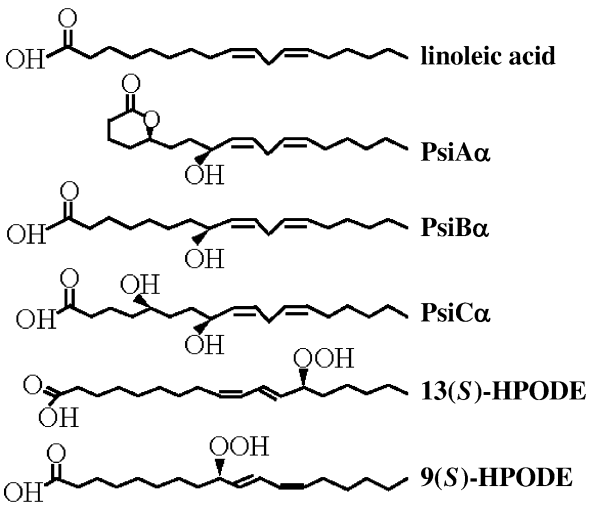


Fig. 2 Chemical structures of linoleic acid-derived oxylipins. PsiAα, PsiBα and PsiCα are *Aspergillus nidulans* oxylipins affecting sporulation. 13(*S*)-HPODE and 9(*S*)-HPODE are linoleic acid derivatives generated by plant lipoxygenases.

detailed below, we now know that oxylipins, in addition to influencing spore production, also regulate secondary metabolite synthesis in *Aspergillus* spp. (Tsitsigiannis and Keller, 2006) and *Fusarium sporotrichiodes* (McDonald *et al.*, 2004).

In *A. nidulans*, the overall oxylipin profile (e.g. relative concentrations of each oxylipin derivative) was reported to regulate the ratio of asexual to sexual spore development (Champe and El-Zayat, 1989). *A. nidulans* generates both sexual (ascospores) and asexual (conidia) spores; ascospores can arise from either homothallic (self) or heterothallic crosses. From studies of *A. nidulans* strains deleted of genes (*ppoA*, *ppoB* and *ppoC*) encoding oxylipin-generating dioxygenases (Tsitsigiannis *et al.*, 2004a,b, 2005b), we now infer that oxylipins generated by Ppo gene products regulate the expression of transcription factors required for meiotic (NsdD) (Chae *et al.*, 1995; Han *et al.*, 2001) and mitotic (BrlA) (Adams *et al.*, 1988; Boylan *et al.*, 1987; Clutterbuck, 1967; Johnstone *et al.*, 1985; reviewed by Adams *et al.*, 1998; Fischer, 2002) sporulation processes. In fact, there is evidence that oxylipins are physically associated with spores: PpoA, which catalyses oxylipin synthesis, is localized to lipid bodies in sexual and asexual fruiting structures (Tsitsigiannis *et al.*, 2004b). Oxylipins are physically associated with reproductive structures in other fungi as well, suggesting a conserved role in development. Immunological analyses provide evidence that oxylipins are localized to asexual reproductive structures (e.g. sporangium, columella and aggregating sporangiospores) in *Pilobolus* and Mucorales, and to sexual structures (e.g. gametangia, asci and the matrix of released aggregating ascospores) in the yeast *Dipodascopsis uninucleata* (e.g. Kock *et al.*, 1998, 2001, 2003; Strauss *et al.*, 2000).

Confirming their importance to oxylipin biogenesis, deletion of any of the three dioxygenase (*ppo*) genes resulted in profound effects on oxylipin profiles (Tsitsigiannis *et al.*, 2004a,b, 2005b). GC-MS analysis of oxylipin profiles from *ppo* mutants revealed that, while PpoA contributes to generation of 8-hydroxy linoleic acid (psiBα, or 8-HODE), PpoB and PpoC are necessary for optimal production of 8-hydroxy oleic acid (psiBβ, or 8-HOE). In addition, PpoC contributes to the formation of prostaglandins from arachidonic acid in *A. nidulans* (Tsitsigiannis *et al.*, 2005a). It is likely that each Ppo enzyme catalyses conversion of more than one oxylipin, as is often observed with fatty acid oxygenases (Su and Oliw, 1996). In *A. nidulans*, mutations in *ppoA* and *ppoB* exert similar effects on sporulation, enhancing the ratio of asexual to sexual spore production, although the Δ*ppoB* mutant is more extreme in this respect. The Δ*ppoC* mutant exhibits the reverse phenotype (an increase in sexual spore production). These effects on sporulation are reflected in expression levels of the sporulation-specific transcriptional regulatory genes, *brlA* and *nsdD* (Tsitsigiannis *et al.*, 2004a, 2005b).

Deletion of *ppo* genes also affected the production of at least three different secondary metabolites in *A. nidulans*, including sterigmatocystin and the antibiotic penicillin (Tsitsigiannis and Keller, 2006), and an octaketide, shamixanthone (J. Frisvald and N.P. Keller, unpublished data). For sterigmatocystin and penicillin, these effects were reflected and supported by levels of biosynthetic gene transcription (Tsitsigiannis and Keller, 2006). The net interpretation of mRNA and chemical analysis is that the PpoB product(s) has a negative role in sterigmatocystin production and the PpoA/PpoC products together positively regulate sterigmatocystin, but negatively regulate penicillin and shamixanthone production. Thus, *ppo* mutations affect the expression of multiple secondary metabolite gene clusters, and these effects vary among individual secondary metabolites.

Plant oxylipins affect sporulation and mycotoxin production

Sporulation and mycotoxin production by *Aspergillus* is affected not only by endogenous oxylipins, but also by plant-derived oxylipins. Seeds are rich in linoleic and linolenic acid, which can be converted to several oxylipin species by plant LOX enzymes. These plant oxylipins are 13*S*-hydroperoxylinoleic/linolenic acid (13*S*-HPODE/TE), and 9*S*-hydroperoxylinoleic/linolenic acid (9*S*-HPODE/TE); their names signify different positions of oxygenation of the carbon chain of the linoleic/linolenic acid precursor. A number of oxylipin products of the plant lipoxygenase pathway have antimicrobial activity, and have been shown to inhibit growth of phytopathogenic fungi (e.g. Hamilton-Kemp *et al.*, 1992; Prost *et al.*, 2005; Vaughn and Gardner, 1993). The specific plant oxylipin derivatives 9*S*-HPODE/TE and 13*S*-HPODE/TE have been shown to alter mycotoxin production and sporulation in *Aspergillus* spp. without obvious effects on growth: exogenous application of pure 13*S*-HPODE to *Aspergillus* cultures repressed aflatoxin and sterigmatocystin gene expression by *A. nidulans* and *A. parasiticus* cultures, whereas pure 9*S*-HPODE had a positive effect (Burow *et al.*, 1997). Filter discs soaked with 0.1 mg (an amount thought to reflect concentrations found in seeds) of 13*S*-HPODE in confluent plate cultures induced conidial development whereas 9*S*-HPODE induced ascospore production. At higher concentrations, 9*S*-HPODE enhanced conidiation (Calvo *et al.*, 1999). Expression of a maize 9-lipoxygenase gene in *A. nidulans* enhanced conidiation in a wild-type background, and restored sterigmatocystin production to oxylipin-deficient *ppoA/ppoC* mutants (M. Brodhagen *et al.*, unpublished data).

Oxylipins in plant–pathogen interactions

Although once thought of as non-responsive to fungal pathogens, living seeds react to fungal infection by altering transcriptional responses (Burow *et al.*, 2000; Tsitsigiannis *et al.*, 2005c; Wilson *et al.*, 2001) leading to biochemical changes in the seed (Burow *et al.*, 2000). Specifically, seed lipoxygenase gene expression is activated or repressed by fungal colonization, leading to changes in levels of bioactive oxylipins (Burow *et al.*, 2000; Tsitsigiannis *et al.*, 2005c; Wilson *et al.*, 2001). Conversely, as outlined above,

seed oxylipins stimulate sporulation and mycotoxin synthesis in *A. nidulans*, *A. flavus* and *A. parasiticus* (Burow *et al.*, 1997; Calvo *et al.*, 1999). Therefore, we postulate that plant oxylipins induce developmental responses in *Aspergillus* and other plant pathogenic fungi by mimicking biological activities of endogenous fungal oxylipins.

Tsitsigiannis and Keller (2006) also showed an association between lipase activity, mycotoxin production and oxylipin concentrations for *A. nidulans*. In their experiments, the Δ*ppoB* mutant showed more lipase activity *in vitro*, produced more sterigmatocystin *in vitro* and appeared visually to macerate peanut seeds more vigorously than the wild-type. By contrast, a *ppoA/ppoC* double mutant presented a near opposite phenotype. Thus, pleiotropic effects of oxylipins may include changes in sporulation, mycotoxigenesis and secreted virulence factors. Whether lipase is involved in a feedback loop with oxylipin production in *A. nidulans* has not been tested.

Broad role for oxylipins in fungal development

Disruption of genes for endogenous oxylipin biosynthesis altered mycotoxin and spore production not only in *A. nidulans* (Tsitsigiannis *et al.*, 2004a,b, 2005b), but also in *A. flavus* (L.R. Milde *et al.*, unpublished data) and *F. sporotrichiodes* (McDonald *et al.*, 2004). In *F. sporotrichioides*, inactivation of a *ppo* homologue led to a decrease in both T2 toxin and sporulation (McDonald *et al.*, 2004). Oxylipins have been chemically isolated from several fungal genera (Bareetseng *et al.*, 2005; Brodowsky and Oliw, 1993; Nakayama *et al.*, 1996; Su and Oliw, 1996), and current genomic data reveal near ubiquity of *ppo* and lipoxygenase genes among filamentous fungi (Tsitsigiannis *et al.*, 2005b), further suggesting a conserved role for oxylipin production. The combined observations from *A. nidulans* and *F. sporotrichiodes* indicate that one such role might be joint regulation of sporulation and secondary metabolism.

Oxylipins as potential GPCR ligands in fungi

As depicted in Fig. 1 and Table 1, sporulation and secondary metabolism are both regulated by components of G protein signalling pathways. For example, mutations in genes for either G protein signal pathway components (e.g. see review by Yu and Keller, 2005) or oxylipin biosynthesis (Tsitsigiannis and Keller, 2006; Tsitsigiannis *et al.*, 2004a, 2005b) result in aberrant transcription of genes governing sporulation and secondary metabolism (e.g. *brlA*, *nsdD* and *aflR*). By extension, it is predicted that future studies will demonstrate involvement of GPCRs in regulation of mycotoxin production. Could GPCRs perceive some of the environmental signals affecting mycotoxin production, including oxylipins? In mammalian systems, oxylipins (e.g. prostaglandins and leukotrienes) are perceived by this class of receptor (e.g. Bos *et al.*, 2003; Tsuboi *et al.*, 2002) as are other fatty acids (e.g. Briscoe *et al.*, 2003). Most compellingly, 9-hydroxyoctadecadienoic acid (9-HODE), a structural analogue of 9*S*-HPODE which changed sporulation and mycotoxin production in *A. nidulans* (Burow *et al.*, 1997; Calvo *et al.*, 1999), was recently shown to bind the mammalian GPCR G2A (Obinata *et al.*, 2005). Whether the fungal G protein signalling pathways governing sporulation and mycotoxin production are triggered by oxylipins remains to be tested.

FUTURE PERSPECTIVES

Secondary metabolite production in fungi is a complex process coupled with morphological development and influenced by environmental conditions (Calvo *et al.*, 2002). Although many elements of molecular regulation of mycotoxin production are known, piecing together the molecular relays that transmit these environmental signals to the nucleus for gene transcription remains work in progress. The molecular regulation surrounding mycotoxin production is perhaps best understood in the model organism *A. nidulans* (e.g. Adams *et al.*, 1998; Calvo *et al.*, 2002; Keller *et al.*, 2005; Yu and Keller, 2005). In *Aspergillus* spp., recent genome sequence and microarray availability may shed additional light on signal transduction pathways and regulatory elements in the future. For example, a comparison of transcripts from aflatoxin-producing and non-producing cultures revealed 753 expressed sequence tags whose expression varied along with aflatoxin production (O'Brian *et al.*, 2003). Genes such as these may represent additional members of a G protein/cAMP/PKA signalling cascade, or ancillary pathways that also affect sterigmatocystin production. For instance, there is evidence that aflatoxin in *A. parasiticus* is regulated by Ca^{2+}/calmodulin-mediated signalling (Jayashree *et al.*, 2000; Praveen Rao and Subramanyam, 1999, 2000). Less pleiotropic regulators may also be identified: in a mutagenesis approach, Butchko *et al.* (1999) isolated *A. nidulans* strains bearing mutations in 19 unlinked loci. These mutants were unable to produce the sterigmatocystin precursor norsolorinic acid, but were apparently unaffected in growth and development, and therefore unlikely to bear mutations in the G protein signalling pathway(s) described above. Especially with the advent of new genome data, components and connections of these signalling pathways regulating mycotoxin production will continue to emerge.

We have presented here evidence implicating an essential role of lipids, and oxylipins in particular, in regulation of mycotoxin and sporulation processes in fungi. Do plant oxylipins induce developmental responses in *Aspergillus* and other plant pathogenic fungi by mimicking biological activities of endogenous fungal oxylipins? Plant oxylipins are similar in structure to those isolated from *Aspergillus* spp. (Fig. 2), suggesting that plant oxylipins might activate fungal receptors, and vice versa, during the *Aspergillus*–seed interaction. The genetic, biochemical and physiological studies reviewed above strongly support a case for conserved oxylipin-mediated signalling between fungus and host

in the *Aspergillus*/seed pathosystem. These studies demonstrated that *Aspergillus* infections induce seed lipoxygenase expression leading to generation of bioactive oxylipins (Burow *et al*., 2000; Tsitsigiannis *et al*., 2005c; Wilson *et al*., 2001); conversely, seed oxylipins stimulate sporulation and mycotoxin synthesis in *A. nidulans*, *A. flavus* and *A. parasiticus* (Burow *et al*., 1997; Calvo *et al*., 1999). Further studies exploring the nature of seed/fungal oxylipin signalling and its role in regulating mycotoxin production may permit interception of such signalling and, thereby, a novel method for preventing mycotoxin contamination of food and feeds.

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