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Pka, Ras and RGS Protein Interactions Regulate Activity of AflR, a Zn(II)2Cys6 Transcription Factor in *Aspergillus nidulans*

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ABSTRACT

Sterigmatocystin (ST) is a carcinogenic polyketide produced by several filamentous fungi including *Aspergillus nidulans*. Expression of ST biosynthetic genes (*stc* genes) requires activity of a Zn(II)2Cys6 transcription factor, AflR. *aflR* is transcriptionally and post-transcriptionally regulated by a G-protein/cAMP/protein kinase A (PkaA) signaling pathway involving FlbA, an RGS (*r*egulator of *G*-protein *s*ignaling) protein. Prior genetic data showed that FlbA transcriptional regulation of *aflR* was PkaA dependent. Here we show that mutation of three PkaA phosphorylation sites in AflR allows resumption of *stc* expression in an overexpression *pkaA* background but does not remediate *stc* expression in a Δ*flbA* background. This demonstrates negative regulation of AflR activity by phosphorylation and shows that FlbA post-transcriptional regulation of *aflR* is PkaA independent. AflR nucleocytoplasmic location further supports PkaA-independent regulation of AflR by FlbA. GFP-tagged AflR is localized to the cytoplasm when *pkaA* is overexpressed but nuclearly located in a Δ*flbA* background. *aflR* is also transcriptionally and post-transcriptionally regulated by RasA. RasA transcriptional control of *aflR* is PkaA independent but RasA post-transcriptional control of AflR is partially mediated by PkaA.

FILAMENTOUS fungi produce a wide range of natural products called secondary metabolites. Interest in these compounds is considerable as many natural products are of medical, industrial, and/or agricultural importance. For example, penicillin and derivatives, produced by Aspergillus, Cephalosporium, and Penicillium species, are widely used as antibiotics (Peñalva *et al.* 1998); lovastatin is a potent cholesterol-lowering drug produced by *Aspergillus terreus* (Kennedy *et al.* 1999); and aflatoxins (AFs), produced by several Aspergillus species, are highly toxic carcinogens contaminating many agricultural crops (Payne 1992). Despite the importance and interest in secondary metabolism, very little is known about its molecular regulation.

The most thorough insight into fungal secondary metabolite regulation arises from studies of the genetic model *A. nidulans*. This organism produces many natural products including sterigmatocystin, the penultimate precursor to aflatoxin B1 (ST; Brown *et al.* 1996), and penicillin (Peñalva *et al.* 1998) and has been used as a heterologous host to study the biosynthesis of other natural products including lovastatin (Kennedy *et al.* 1999). Critical advances in our understanding of fungal secondary metabolism include the discovery of penicillin (Montenegro *et al.* 1992) and ST biosynthetic gene clusters (Brown *et al.* 1996) and the discovery of a G-protein-mediated growth pathway in *A. nidulans* regulating secondary metabolism production (Hicks *et al.* 1997; Tag *et al.* 2000). It is now apparent that structural genes required for secondary metabolite production are usually clustered (Keller and Hohn 1997), that the regulation of the clustered genes is largely dependent on pathway-specific transcription factors (Fernandes *et al.* 1998; Hohn *et al.* 1999; Tsuji *et al.* 2000), and that G-protein regulation of fungal secondary metabolism is likely to be a conserved phenomenon (Tag *et al.* 2000).

The transcription factor responsible for regulating the ST/AF gene cluster is a Zn(II)2Cys6 binuclear cluster protein encoded by *aflR* (Fernandes *et al.* 1998). Deletion of this gene results in elimination of *stc* (ST cluster) gene expression and subsequent ST production. A series of studies have shown that *aflR* expression is regulated by G-protein/cAMP/protein kinase A-mediated signaling (Hicks *et al.* 1997; Shimizu and Keller 2001; Figure 1). In this pathway, when a G-protein α-subunit, FadA, and/or a protein kinase A catalytic subunit, PkaA, are active, *aflR* expression and subsequent *stc* expression and ST production are blocked. FlbA, a protein containing an RGS (*r*egulator of *G*-protein *s*ignaling) domain, is also required for ST biosynthesis, in part via enhancing the intrinsic GTPase activity of FadA (Hicks *et al.* 1997). Loss-of-function *flbA* (Δ*flbA*) mutants exhibit a loss of *aflR* and *stc* expression and ST production.

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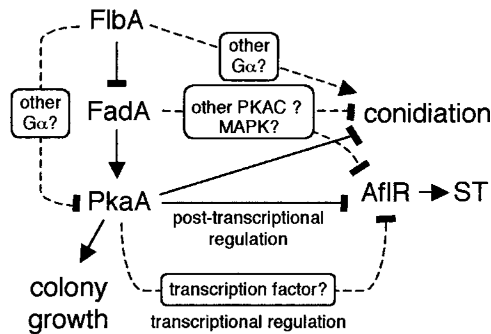


Figure 1.—The proposed model of G-protein signal transduction pathway regulating ST production and morphogenesis in *A. nidulans* (Shimizu and Keller 2001).

The mechanism by which FlbA, FadA, and PkaA regulate *aflR* is complex. Deletion of *pkaA* cannot restore *aflR* and *stc* expression in a genetic background containing a *fadA*^{G42R} allele (an activated form of FadA) but can restore expression of *aflR* and *stc* genes in a Δ*flbA* background (Shimizu and Keller 2001). Also, ST production is not reestablished when *aflR* is overexpressed in Δ*flbA* or a *pkaA* overexpression background (Shimizu and Keller 2001). However, ST production is remediated when *aflR* is overexpressed in the *fadA*^{G42R} background (J. K. Hicks and N. P. Keller, unpublished data). These data support a model of PkaA-dependent- and FadA-independent-FlbA transcriptional and post-transcriptional regulation of *aflR*.

In this study we attempt to clarify the relationship between FlbA and PkaA in controlling AflR and to elucidate the mechanism of post-transcriptional regulation of AflR. Because we identified three putative PKA-specific phosphorylation motifs in AflR, we hypothesized that PkaA might inactivate AflR activity by phosphorylation and that FlbA regulation of AflR was mediated via such phosphorylation. As shown here, AflR is inactivated by PkaA phosphorylation, but this is not the mechanism by which FlbA regulates AflR.

Additionally we explore the relationship between RasA, a member of the family of small GTP-binding proteins, and PkaA in regulating *aflR*. Preliminary data showed that ST biosynthesis was blocked in *A. nidulans* mutants producing a dominant active form of RasA, RasA^{G17V} (K. Shimizu and N. P. Keller, unpublished data). Although we have recently shown that RasA and cAMP-dependent PkaA signaling proceed independently in controlling asexual spore germination in *A. nidulans* (Fillinger *et al.* 2002), we did not examine whether there could be any interaction between RasA and the cAMP-dependent pathway in regulating ST production. Examination of the relationship between Ras and cAMP-dependent signaling in other fungi indicates a role for both dependent and independent signaling. For example, in *Cryptococcus neoformans*, Ras1 signals in part through a cAMP-dependent cascade to regulate mating, filamentation, and growth at high temperature (Alspaugh *et al.* 2000). However, a *ras1* mutant strain of the same fungus was not deficient in melanin or capsule production, two cAMP-dependent phenotypes (Alspaugh *et al.* 1997). Here we show that RasA transcriptionally and post-transcriptionally controls *aflR* activity and that the latter but not the former control is mediated through *pkaA*.

MATERIALS AND METHODS

Strains and growth conditions: *A. nidulans* strains used in this study are listed in Table 1. TJH119.3 is a transformant of RJH288 with pJH119. TJH120.K3 is a transformant of FGSC237 with pJH120. RKIS33.9 was obtained by sexual recombination between TJH120.K3 and RJH254. DKIS4 and DKIS5 are diploids of RKIS33.9 with RJH276 and TMF4.12, respectively. DKIS6 and DKIS7 are diploids of TJH119.3 with RJH276 and TMF4.12, respectively. TKIS40.2 and TKIS33.4 are transformants of FGSC237 with plasmids pKIS40 or pKIS33, respectively, and recombinant strains obtained from crosses with RJH254 are designated as RKIS42.17 and RKIS41.23, respectively. DKIS14 and DKIS15 are diploids of RKIS42.17 with RJH276 and TMF4.12, respectively. DKIS12 and DKIS13 are diploids of RKIS41.23 with RJH276 and TMF4.12, respectively. RKIS34.8, RKIS46.1, RPFR3.2, and RPFR2.3 are progeny of crosses between TBN39.5 and RKIS33.9, TJH119.3, RKIS42.17, or RKIS41.23, respectively. RKIS1 is a recombinant from FGSC26 crossed to FGSC237 and RKIS28.5 is a progeny of AST27 crossed to RKIS1. RTPH1.2 is a progeny of the cross between AST27 and RDIT1.1. RTPH2.3, RTPH3.2, and RTPH4.1 are the progeny of the cross between RTPH1.2 and RKIS33.9, RKIS38.8, and RKIS42.17, respectively. RTPH6.2 is a progeny of the cross between RKIS13.7 and RTPH2.3. RTPH10.1 is a progeny of the cross between RJH254 and TJH120.K3; RTPH11.3 is a progeny between the cross of RKIS37.5 and RKIS37.18. Aspergillus minimal medium with 1% glucose (MMG) was used unless stated otherwise. Glucose was replaced with 100 mm threonine for overexpression experiments (MMT).

Plasmid construction: pJH111 containing a 2.5-kb *Apa*I fragment containing the *aflR* gene inside the multiple cloning site of pBluescript (SK$^-$) was used for site-directed mutagenesis (Kunkel *et al.* 1991). Primers 5′-CCCAAGACAGTCGTAGAG CTGCAGTCTCTTCCTTTGAGG-3′ and 5′-AATCTATTCGC CGCCGCGCCGCGAGCAGCTCCAGCTCTGCCTCTAGTA-3′ were used to introduce S323A or S381,382A mutations, respectively, by site-directed mutagenesis. The resulting plasmid pJH113.4 contains the S323A mutation, pJH109 contains the S381,382A mutations, and pJH112 contains the S323,381,382A mutations. Using the primers 5′-CATATGCAAGCTTCATGG AGCCCC-3′ and 5′-GACAGAGGTACCGAGGCCGAC-3′, PCR fragments were then obtained from pJH111, pJH113.4, pJH109, or pJH112 and introduced into the *Hin*dIII-*Kpn*I sites of pCN2 to create pJH120, pJH118, pJH117, and pJH119, respectively. pCN2 contains a 1.75-kb fragment containing the 5′ portion of the *trpC* gene, which can complement *trpC801* mutation by single crossing over, and the *alcA*(*p*) for artificial overexpression of a fusion gene.

pKIS40 and pKIS33 were constructed for synthetic green fluorescent protein (sGFP)-AflR fusion protein expression *in vivo*. A fragment encoding sGFP was amplified with primers 5′-GGA AGCTTCCATGGTGAGCAAG-3′ and 5′-CCCCAAGCTTTTG TACAGCTC-3′ by using pPRgfT4 (kindly provided by C. Cortez) as a template DNA, digested by *Hin*dIII, and cloned into the *Hin*dIII site of pJH120 or pJH119 to create pKIS40 or pKIS33, respectively. pKIS26 was constructed by introducing a PCR fragment containing *aflR* derived from pJH111 with

the primers 5′-TTTCCATGGAGCCCCCAGCGA-3′ and 5′-AAGGATCCGAG-CGTGGCGGA-3′ into the *Nco*I-*Bam*HI sites of pET27b (Novagen). pKIS26 expresses the AflR-6xHis Tag fusion protein when induced by IPTG. pKIS27 was constructed by introducing a PCR fragment containing *aflR* derived from pJH112 with the primers 5′-TTTCCATGGAGCCCCCAGCGA-3′ and 5′-AAGGATCCGAG-CGTGGCGGA-3′ into the *Nco*I-*Bam*HI sites of pET27b (Novagen, Cambridge, MA) to allow expression of the S323,381,382A AflR protein. All the plasmids were maintained in DH5α, and pKIS26 was transformed into BL21(DE3) (Novagen) for appropriate expression in *Escherichia coli*.

Protein purification: Five milliliters of an overnight culture of BL21 DE3 carrying pKIS26 was transferred to 100 ml of Luria-Bertani medium and grown until the OD_{600} reached 0.6. The bacterial cells were harvested and lysed in 100 mm NaH_2PO_4, 10 mm Tris-HCl, 6 m guanidine hydrochloride (pH 8.0) at room temperature with shaking. The debris was removed by centrifugation at 9000 × *g* for 30 min, and cleared lysate was obtained. The fusion protein was purified with a Ni-NTA agarose gel (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The fractions containing the fusion protein were dialysed to refold the protein by stepwise dialysis using the following buffer: 30 mm Tris-HCl (pH 7.6), 200 mm KCl, 1 mm EDTA, 5 mm dithiothreitol (DTT), 10% (v/v) glycerol, containing 6 m, 3 m, 2 m, 0.5 m, and 0 m urea. The purified protein was confirmed by Western blot with an anti-His antibody (QIAGEN).

Pka phosphorylation: The recombinant AflR protein was added to a Pka reaction buffer (20 mm HEPES, 5 mm $MgCl_2$, 5 mm DTT, 1 mm ATP, 100 mm NaCl, 0.01 mCi [^{32}P]ATP, pH 7.5). Fifty units of Pka (Sigma, St. Louis) were added to the reaction mixture and incubated at 30° for 30 min. Pka inhibitor (1 μg; Sigma) was added to the negative control reaction.

RNA manipulation: MMG (500 ml) was inoculated with 5×10^8 spores and incubated with shaking at 37° for 14 hr. Then the mycelia were harvested, washed with H_2O, and transferred into either MMG or MMT and incubated under the same conditions. After designated incubation times after shift (6 and 12 hr), the mycelia were harvested and lyophilized. Total RNA was extracted from the dried mycelia with Trizol (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's direction. Ten micrograms of total RNA from each sample was used for mRNA analysis. A 1.5-kb *Bam*HI fragment of pKIS17, a 1.3-kb *Eco*RV-*Xho*I fragment of pAHK25, a 0.75-kb *Sac*II-*Sma*I fragment from pRB7, and a 0.7-kb *Bam*HI fragment of pRASC23R were used as *pkaA*-, *aflR*-, *stcU*-, and *rasA*-specific probes, respectively.

Chromatography: ST was extracted and analyzed by thin layer chromatography as described by Shimizu and Keller (2001).

Microscopy: Fungal spores were inoculated in MMG, grown, and then transferred to MMT as described above for RNA manipulation. After additional 6-hr incubation in MMT with shaking at 37°, the mycelia were harvested. GFP was visualized without any treatment, and nuclei were strained by addition of 4′,6-diamidino-2-phenylindole (DAPI) DNA dye to the mycelial samples to the final concentration of 0.1 μg/ml and incubated at room temperature for 5 min before observation. Cells were viewed using an Olympus BX60F-3 fluorescent microscope with a standard FITC filter for GFP and a DAPI optimized filter for DAPI stain, and images were scanned through Magnafire digital camera (Olympus) and transferred to Adobe Photoshop 5.5.

RESULTS

AflR protein is a target for phosphorylation by Pka *in vitro*: The *A. nidulans* AflR protein has three conserved Pka-specific phosphorylation motifs (data not shown). To determine if AflR is phosphorylated by Pka, we constructed a plasmid, pKIS26, carrying the coding sequence for the AflR-6xHis Tag fusion protein. We expressed and purified this protein in *E. coli* as described in materials and methods. As expected, we got a protein of the predicted size (∼53 kD) which was visualized by both Commassie staining (data not shown) and Western blot analysis (Figure 2). Because the fusion protein was found in the inclusion bodies and could not be denatured even with 8 m urea, guanidine hydrochloride was used to solubilize the protein. After Ni-NTA agarose gel purification, the protein was dialysed to renature it. The AflR-6xHis Tag fusion protein was incubated with Pka and [α-^{32}P]ATP. The reaction with the fusion protein and Pka resulted in a ^{32}P-labeled ∼53-kD protein (Figure 2). Phosphorylation of AflR was inhibited by adding a Pka inhibitor. We also prepared reaction mixtures lacking either Pka, the fusion protein, or [α-^{32}P]ATP. None of these reactions yielded an ∼53-kD ^{32}P-labeled protein. The Western blot analysis of the same samples showed the presence of AflR at 53 kD.

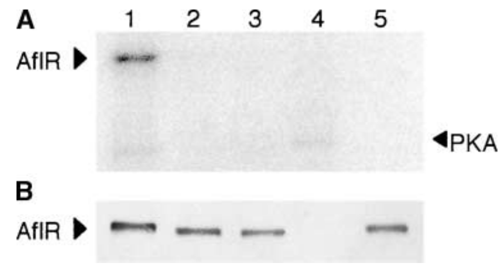


Figure 2.—AflR protein is phosphorylated by Pka *in vitro*. AflR was expressed in *E. coli*, purified, and used in a Pka phosphorylation assay. Note that Pka has autophosphorylation motifs that can be phosphorylated. (A) Autoradiography; (B) Western blot with anti-6xHis antibody. Lane 1, AflR + Pka + [^{32}P]ATP; lane 2, AflR + Pka + [^{32}P]ATP + Pka inhibitor; lane 3, AflR + [^{32}P]ATP; lane 4, Pka + [^{32}P]ATP; and lane 5, AflR + Pka.

Mutations in Pka phosphorylation sites allow AflR activity in a Pka overexpression background: We have observed that overexpression of *aflR* in a *pkaA* overexpression background does not allow *stc* gene expression (Shimizu and Keller 2001), but overexpression of *aflR* in a wild-type *pkaA* background results in enhanced *stcU* gene expression (Hicks *et al.* 1997). This implies a post-transcriptional regulation of AflR by PkaA. We asked if the negative regulation of AflR by overexpression of *pkaA* was a consequence of phosphorylation of AflR protein. We designed two *aflR* mutant alleles in which one ($aflR^{S323A}$) or all three ($aflR^{S323AS381AS382A}$) putative phosphorylation sites were mutated. Overexpression constructs bearing these alleles were introduced into both a wild-type *pkaA* and a *pkaA* overexpression background (Table 1). Figure 3A shows the results of gene expression in the $aflR^{S323AS381AS382A}$ background *vs.* wild type. Both *pkaA* and *aflR* alleles were overexpressed when grown in MMT, an *alcA*(*p*) activat-

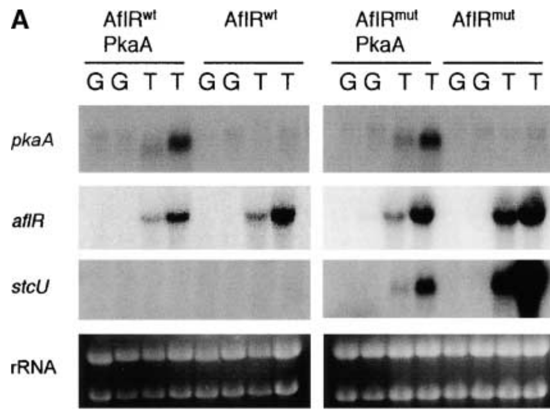
TABLE 1

Fungal strains used in this study

Strain	Genotype	Source
FGSC237	*pabaA1, yA2; trpC801*	FGSC
FGSC26	*biA1*	FGSC
TMF4.12	*biA1; argB2; methG1, ΔaflR::argB*	Fernandes *et al.* (1998)
TBN39.5	*biA1, ΔflbA::argB; methG1*	Lee and Adams (1994)
RJH276	*biA1; methG1, ΔaflR::argB; alcA(p)::pkaA::trpC*	This study
RKIS33.9	*pabaA1, biA1, yA2; ΔaflR::argB; alcA(p)::aflR::trpC*	This study
TJH118.3	*pabaA1, biA1, yA2; ΔaflR::argB; alcA(p)::aflR*^{S323A}*::trpC*	This study
TJH119.3	*pabaA1, biA1, yA2; ΔaflR::argB; alcA(p)::aflR*^{S323,381,382A}*::trpC*	This study
TJH120.K3	*pabaA1, yA2; alcA(p)::aflR::trpC*	This study
RJH254	*biA1; argB2; methG1, ΔaflR::argB; trpC801*	This study
RJH288	*pabaA1, biA1, yA2; ΔaflR::argB; trpC801*	This study
TKIS40.2	*pabaA1, yA2; alcA(p)::sgfp-aflR::trpC*	This study
TKIS33.4	*pabaA1, yA2; alcA(p)::sgfp-aflR*^{S323,381,382A}*::trpC*	This study
RKIS41.23	*pabaA1, biA1, yA2; ΔaflR::argB; alcA(p)::sgfp-aflR*^{S323,381,382A}*::trpC*	This study
RKIS42.17	*pabaA1, biA1, yA2; ΔaflR::argB; alcA(p)::sgfp-aflR::trpC*	This study
RKIS34.8	*biA1, yA2, ΔflbA::argB; ΔaflR::argB; alcA(p)::aflR::trpC*	This study
RKIS46.1	*biA1, yA2, ΔflbA::argB; ΔaflR::argB; alcA(p)::aflR*^{S323,381,382A}*::trpC*	This study
RPFR3.2	*biA1, yA2, ΔflbA::argB; ΔaflR::argB; alcA(p)::sgfp-aflR::trpC*	This study
RPFR2.3	*biA1, yA2, ΔflbA::argB; ΔaflR::argB; alcA(p)::sgfp-aflR*^{S323,381,382A}*::trp*	This study
AST27	*biA1; alc*A*(p)::ras*A^{G17V}*::arg*B	Som and Kolaparthi (1994)
RDIT1.1	*pyrG89; argB2; methG1*	D. Tsitsigiannis
RKIS1	*pabaA1, yA2*	This study
RKIS28.5	*pabaA1, yA2, alc*A*(p)::ras*A^{G17V}*::arg*B	This study
RTPH2.3	*biA1; alc*A*(p)::ras*A^{G17V}*::arg*B, *ΔaflR::arg*B, *alc*A*(p)::aflR::trpC*	This study
RTPH6.2	*biA1; ΔpkaA::argB; alc*A*(p)::ras*A^{G17V}*::arg*B, *ΔaflR::argB; alcA(p)::aflR::trpC*	This study
RTPH10.1	*biA1; ΔaflR::argB, alcA(p)::aflR::trpC*	This study
RTPH3.2	*biA1; alc*A*(p)::ras*A^{G17V}*::arg*B, *ΔaflR::arg*B, *alc*A*(p)::aflR*^{S323,381,382A}*::trpC*	This study
RTPH11.3	*biA1; ΔaflR::argB, alcA(p)::aflR*^{S323,381,382A}*::trpC*	This study
RTPH1.2	*biA1; meth*G1; *alc*A*(p)::ras*A^{G17V}*::arg*B	This study
RTPH4.1	*biA1; alc*A*(p)::ras*A^{G17V}*::arg*B; *ΔaflR::argB; alcA(p)::sgfp-aflR::trpC*	This study
RKIS13.7	*pabaA1, yA2, ΔpkaA::arg*B, *alc*A*(p)::ras*A^{G17V}*::arg*B	This study
RKIS38.8	*biA1, pabaA1, yA2, ΔaflR::argB, alcA(p)::aflR*$^{S323.381,382A}$*::trpC*	This study
RKIS37.5	*biA1, methG1, wA3, ΔaflR::argB, alcA(p)::aflR*$^{S323.381,382A}$*::trpC*	This study
RKIS37.18	*pabaA1, biA1,yA2, ΔaflR::argB*	This study
DKIS4	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::aflR::trpC* *+, biA1, +; methG1, ΔaflR::argB; alcA(p)::pkaA::trpC*	This study
DKIS5	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::aflR::trpC* *+, biA1, +; methG1, ΔaflR::argB; +*	This study
DKIS6	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::aflR*^{S323,381,382A}*::trpC* *+, biA1, +; methG1, ΔaflR::argB; alcA(p)::pkaA::trpC*	This study
DKIS7	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::aflR*^{S323,381,382A}*::trpC* *+, biA1, +; methG1, ΔaflR::argB; +*	This study
DKIS14	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::sgfp-aflR::trpC* *+, biA1, +; methG1, ΔaflR::argB; alcA(p)::pkaA::trpC*	This study
DKIS15	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::sgfp-aflR::trpC* *+, biA1, +; methG1, ΔaflR::argB; +*	This study
DKIS12	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::sgfp-aflR*^{S323,381,382A}*::trpC* *+, biA1, +; methG1, ΔaflR::argB; alcA(p)::pkaA::trpC*	This study
DKIS13	*pabaA1, biA1, yA2; +, ΔaflR::argB; alcA(p)::sgfp-aflR*^{S323,381,382A}*::trpC* *+, biA1, +; methG1, ΔaflR::argB; +*	This study

Strain starting with "T" is an original transformant, "R" is a recombinant, and "D" is a diploid. Other strains are designated as explained in source column. All strains contain the *veA1* allele. FGSC, Fungal Genetics Stock Center.

ing medium. However, *stcU* was expressed only in the *pkaA* overexpression strain containing the *aflR*^{S323AS381AS382A} allele. ST production reflected levels of *stcU* expression (Figure 3B). *stcU* expression in the *aflR*^{S323A} background was not as strong as that of the *aflR*^{S323AS381AS382A} background yet was greater than that of wild type (data not shown). This may indicate a dosage effect of phosphorylation on AflR activity.



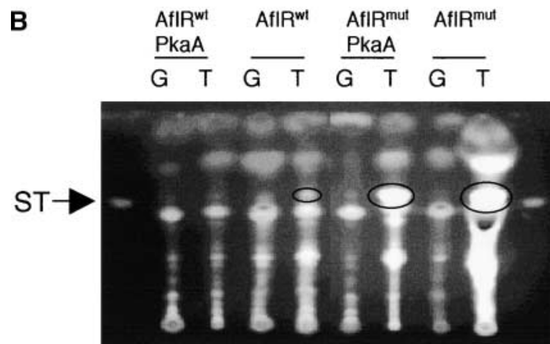


Figure 3.—Mutation of Pka phosphorylation sites in AflR remediates *stcU* expression in *pkaA* overexpression strains. (A) Total RNA was probed with *pkaA*-, *aflR*-, or *stcU*-specific probes. Lanes 1–4, *pkaA* and *aflR* overexpression strain DKIS4; lanes 5–8, *aflR* overexpression strain DKIS5; lanes 9–12, *pkaA* and *aflR*^{S323,381,382A} overexpression strain DKIS6; and lanes 13–16, *aflR*^{S323,381,382A} overexpression strain DKIS7. Although no *stcU* is observed in lanes 7 and 8 in this exposure, the gene is expressed as reflected by ST production in the TLC (B) and overexposed film (data not shown). Lanes 1, 5, 9, and 13 were in G (noninducing conditions) for 6 hr and lanes 2, 6, 10, and 14, for 12 hr; lanes 3, 7, 11, and 15 were in T (inducing conditions) for 6 hr and lanes 4, 8, 12, and 16, for 12 hr. (B) Extracts of the same strains from a 72-hr time point run on a TLC plate. ST spots are circled to distinguish them from other compounds with similar hue in the black-and-white picture. ST standards are on the first and last rows and an arrow indicates one standard.

The *aflR*^{S323AS381AS382A} allele was also expressed as a 6xHis Tag fusion protein. This fusion protein, like wild-type AflR, was also ^{32}P-labeled by Pka (data not shown) under our conditions. Whether this means that AflR contains noncanonical PKA phosphorylation sites or that phosphorylation can occur *in vitro* but not *in vivo* is not known at this time. It is also possible that the Pka used in our assay (from bovine heart; Sigma) may interact with AflR differently than *A. nidulans* PkaA.

PkaA overexpression reduces localization of AflR to the nucleus: On the basis of studies in other organisms we considered it likely that the inability of AflR to function in a *pkaA* overexpression strain could be due to mislocalization, shortened half-life of the protein, and/or inability to bind to *stc* promoters (Whitmarsh and Davis 2000). To address the first possibility, *gfp::aflR* and *gfp::aflR*^{S323AS381AS382A} alleles were placed in wild-type and overexpression *pkaA* backgrounds. First we ascertained that these alleles functioned properly by placing them in a Δ*aflR* background and then we determined that both *stcU* expression and ST production in these strains was no different from that in strains containing *aflR* alleles lacking GFP (data not shown).

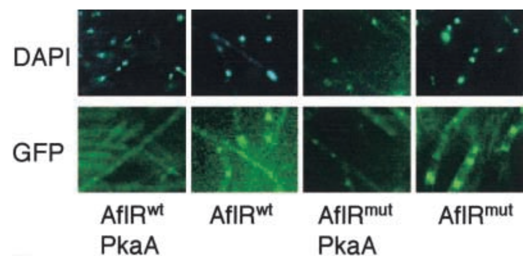


Figure 4.—sGFP-AflR distribution in PkaA and AflR mutant backgrounds. The sGFP-AflR fusion proteins were expressed and stained with DAPI. AflR^{wt}; PkaA indicates *pkaA* and *aflR* overexpression strain DKIS14; AflR^{wt} indicates *aflR* overexpression strain DKIS15; AflR^{mut}, PkaA indicates *pkaA* and *aflR*^{S323,381,382A} overexpression strain DKIS12; and AflR^{mut} indicates *aflR*^{S323,381,382A} overexpression strain DKIS13.

Overexpression of *pkaA* reduced the level of AflR protein in the nucleus regardless of whether a wild-type or *aflR*^{S323AS381AS382A} allele was present (Figure 4). However, in contrast to the strain with the wild-type allele, some nuclear localization of GFP could still be detected in the *pka* overexpression strain with the mutant *aflR* allele, although to a lesser extent than in a wild-type *pkaA* background (Figure 4). An examination of nuclei in a single microscope field showed that ∼50% of the nuclei in wild-type *pkaA* strains and none of nuclei in *pkaA* overexpression strains showed intense accumulation of GFP. This strongly implicates the importance of PkaA activity in controlling high AflR levels in the nucleus. Whether the absence of nuclear AflR in the *pkaA* overexpression background is due to inability of AflR to arrive in the nucleus or inability to reside in the nucleus after arrival is unknown.

FlbA requirement for AflR activity is not mediated by PkaA: FlbA is an RGS domain protein that functions in negatively regulating FadA, a Gα-subunit. When FadA is active (or FlbA is inactive), it indirectly activates PkaA, resulting in repression of *aflR* transcription and AflR activity with subsequent loss of *stc* gene expression (Hicks *et al.* 1997; Shimizu and Keller 2001). However, *stc* gene expression was observed when *aflR* was overexpressed in a constitutively activated FadA (FadA^{G42R}) background but not in a Δ*flbA* background, suggesting a post-transcriptional regulation of AflR by FlbA that is independent of FadA (J. K. Hicks and N. P. Keller,

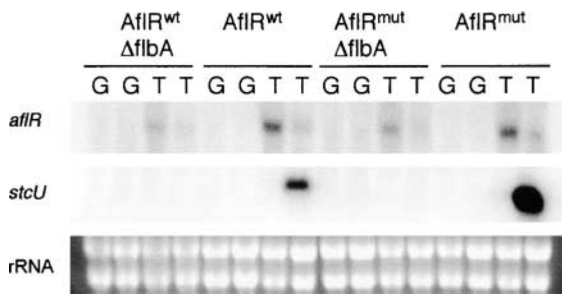


FIGURE 5.—Mutations in AflR do not restore *stcU* expression in the Δ*flbA* background. Total RNA was probed with *aflR*- or *stcU*-specific probes. Lanes 1–4, Δ*flbA* and *aflR* overexpression strain RKIS34.8; lanes 5–8, *aflR* overexpression strain RIS33.9; lanes 9–12, Δ*flbA* and *aflR*^{S323,381,382A} overexpression strain RKIS46.1; and lanes 13–16, *aflR*^{S323,381,382A} overexpression strain TJH119.3. Lanes 1, 5, 9, and 13 were in MMG (noninducing conditions) for 6 hr and lanes 2, 6, 10, and 14, for 12 hr; lanes 3, 7, 11, and 15 were in MMT (inducing conditions) for 6 hr and lanes 4, 8, 12, and 16, for 12 hr.

unpublished data; Figure 5). Additionally, epistasis studies have indicated that the requirement of FlbA for *aflR* expression might be mediated through *pkaA*, as the Δ*flbA;* Δ*pkaA* double mutant was restored for ST production (Shimizu and Keller 2001).

Here we asked if PkaA has a role in FlbA post-transcriptional regulation of AflR. We reasoned that if the post-transcriptional regulation of AflR by FlbA occurs through PkaA phosphorylation of AflR, then overexpression of the *aflR*^{S323AS381AS382A} allele in a Δ*flbA* background would restore *stcU* gene expression. Figure 5 illustrates that *stcU* was not rescued in this strain, as opposed to when *aflR*^{S323AS381AS382A} is overexpressed in a *pkaA* overexpression background (Figure 3A).

Examination of GFP-tagged AflR also supported a PkaA-independent role for FlbA control of AflR activity. In both the wild-type GFP::aflR and GFP::AflR^{S323AS381AS382A} strains, microscopic examination revealed that the percentage of GFP nuclei (counts of 50) was identical regardless of the presence of a wild-type *flbA* or Δ*flbA* background (Figure 6). Unlike PkaA, FlbA is not necessary for localization of AflR to the nucleus but likely acts through another protein(s) to affect AflR activity within the nucleus (we think the increased fluorescence in the Δ*flbA* strain does not indicate increased nuclear AflR content in this background but cannot rule out this possibility). Together, the data in this section suggest that the post-transcriptional regulation of AflR by FlbA does not occur through PkaA.

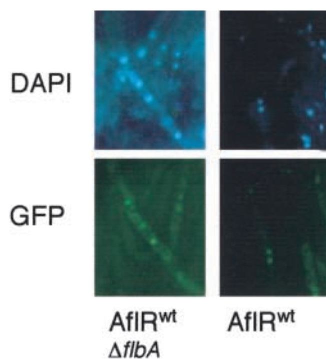


FIGURE 6.—sGFP-AflR distribution is located in the nucleus in the Δ*flbA*, *OE::aflR* background. Cells were stained with DAPI or monitored for sGFP-AflR fusion protein expression. AflR^{wt}, Δ*flbA* indicates Δ*flbA* and *aflR* overexpression strain RPFR3.2. AflR^{wt} indicates *aflR* overexpression strain RKIS-42.17.

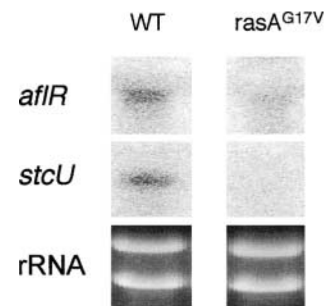


FIGURE 7.—Expression of *aflR* is repressed when RasA is active. Total RNA was probed with *aflR*- or *stcU*-specific probes. Lane 1, wild-type strain RKIS1 in MMT for 12 hr; lane 2, *alcA(p)*::*rasA*^{G17V} strain RKIS28.5 in MMT for 12 hr.

Post-transcriptional but not transcriptional regulation of AflR by RasA is mediated through Pka: Our initial examination of a RasA mutant, RasA^{G17V}, where RasA is locked in the active GTP-bound state (Som and Kola-

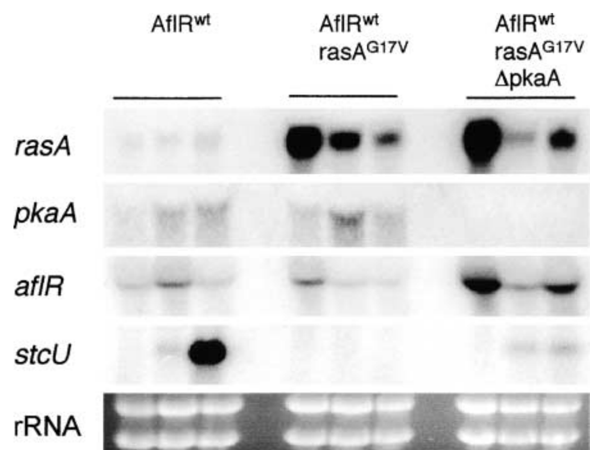


FIGURE 8.—The sterigmatocystin biosynthetic gene *stcU* is not expressed in a *rasA*^{G17V}*; OE::aflR* strain, but is expressed when combined with Δ*pkaA*. Total RNA was extracted after shift to MMT and probed with *rasA*-, *pkaA*-, *aflR*-, or *stcU*-specific probes. Lanes 1–3, wild-type strain RTPH10.1; lanes 4–6, *rasA*^{G17V} strain RTPH2.3; and lanes 7–9, *rasA*^{G17V}*;* Δ*pkaA* strain RTPH6.2. All the strains tested carried *alcA(p)*::*aflR*. In each data set from left to right, samples were prepared from mycelia grown for 2, 6, or 12 hr after shift.

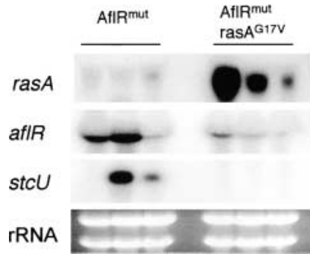


Figure 9.—The sterigmatocystin biosynthetic gene *stcU* is not expressed in a *rasA*^{G17V}*; OE::aflR*^{S323,381,382A} strain. Total RNA was extracted after shift to MMT and probed with *rasA*-, *pkaA*-, *aflR*-, or *stcU*-specific probes. Lanes 1–3, wild-type strain RTPH11.3 and lanes 4–6, *rasA*^{G17V} strain RTPH3.2. All the strains tested carried *alcA*(*p*)::*aflR*^{S323,381,382A}. In each data set from left to right, samples were prepared from mycelia grown for 2, 6, or 12 hr after shift.

parthi 1994), suggested that RasA negatively regulated ST biosynthesis. This was confirmed here as shown in Figures 7 and 8 where expression of *rasA*^{G17V} results in both transcriptional and post-transcriptional regulation of *aflR*. Figure 7 shows that *aflR* transcript is reduced in a *rasA*^{G17V} background, and Figure 8 shows that forced expression of *aflR* cannot remediate *stcU* expression in the *rasA*^{G17V} background (lanes 4–6).

Investigations in other fungi have shown that RAS protein can signal through the cAMP/Pka pathway (Kataoka *et al.* 1984; Toda *et al.* 1985; Alspaugh *et al.* 2000). We therefore thought it possible that there could be an interaction between RasA and PkaA in some aspect of Aspergillus development, such as secondary metabolism, on the basis of the observations in other fungi. We first asked if AflR phosphorylation was important for AflR activity in the RasA^{G17V} background by overexpressing *aflR*^{S323,381,382A} in a RasA^{G17V} background. *stcU* expression was not seen in this strain (Figure 9), indicating that RasA repressed AflR activity post-transcriptionally, regardless of the phosphorylation state of AflR.

Next we introduced the Δ*pkaA* allele into both the *rasA*^{G17V} and the *rasA*^{G17V}; *aflR* overexpression backgrounds. *stcU* transcription was partially remediated in the Δ*pkaA*; *rasA*^{G17V}; *aflR* overexpression strain (Figure 8, lanes 7–9) and not detected in the Δ*pkaA*; *rasA*^{G17V} strain (data not shown). These data suggest that PkaA activity is partially required for RasA post-transcriptional control of AflR but that this control is not associated with phosphorylation of AflR (Figure 9). On the other hand, RasA transcriptional regulation of *aflR* expression is PkaA independent.

We also placed the *gfp::aflR* construct in the RasA^{G17V} background (strain RTPH4.1) to observe the subcellular localization of the AflR protein. We found that the AflR protein localizes in the nucleus, athough not to the intensity of the wild-type control, RKIS42.17 (data not shown). This finding was similar to the observed GFP::AflR localization in the Δ*flbA* strain (Figure 6) in which the AflR protein was in the nucleus but not functional.

DISCUSSION

In this study, we have demonstrated the complex regulation of toxin production in the filamentous fungus *A. nidulans* via the ST/AF-specific transcription factor AflR. We have chosen *A. nidulans* as a model system to elucidate the mechanism(s) of gene regulation involved in secondary metabolism because (1) the related compound, AF, produced by *A. flavus* and *A. paraciticus* is one of the most threatening carcinogens worldwide; (2) Aspergillus spp. closely related to *A. flavus* and containing the aflatoxin gene cluster are frequently used for food fermentation in many countries; and (3) basic genetic knowledge of secondary metabolism and molecular genetic techniques are well developed for *A. nidulans.* We have focused on the regulation of AflR because it is essential for ST and AF biosynthetic gene expression (Chang *et al.* 1993; Woloshuk *et al.* 1995; Yu *et al.* 1996; Fernandes *et al.* 1998) and representative of Zn(II)2-Cys6 binuclear cluster transcription factors (Todd and Andrianopoulos 1997).

Previous research suggested the existence of a Pka-mediated post-transcriptional regulation mechanism to control AflR activity (Shimizu and Keller 2001). The presence of three Pka-specific phosphorylation motifs in AflR suggested that AflR might be post-transcriptionally regulated through direct phosphorylation by PkaA, the Pka catalytic subunit. This hypothesis was supported by two pieces of evidence as shown here. One was that AflR is phosphorylated by PkaA *in vitro* (Figure 2). The assay we employed did not precisely identify which amino acids are phosphorylated by PkaA. However, the results from examination of two *aflR* alleles with different number of modified Pka sites suggest that all three serine residues are phosphorylated as there was an increase in *stcU* expression and ST production with each successive modification. The remediation of AflR activity in the proteins lacking serine residues (Figure 3) presented the second piece of evidence that PkaA directly phosphorylated AflR.

The mutations introduced in the Pka phosphorylation motifs resulted in a great increase in AflR activity that was reflected in enhanced *stcU* expression and overproduction of ST in the AflR^{S323AS381AS382A} strains. This enhanced *stcU* expression and ST production was even more pronounced when the mutated *aflR* allele was overexpressed in a wild-type *pkaA* background (Figure 3A, lanes 15 and 16). This latter observation suggests that PkaA post-transcriptionally regulates AflR not only by direct phosphorylation but also through regulation of a mediating protein(s) that also regulates AflR. One candidate protein is LaeA, a nuclear protein that is required for AflR activity and is itself negatively regulated by PkaA (J.-W. Bok and N. P. Keller, unpublished data). Yeast transcription factors have been found to be directly and indirectly regulated by phosphorylation activities of Pka. For example, the *Schizosaccharomyces*

pombe Rst2 protein involved in sexual development and gluconeogenesis is phosphorylated by Pka as well as by an additional factor(s) subject to Pka regulation (Higuchi *et al.* 2002). Thus, concomitant regulation of transcription factors by Pka and its associated target proteins may be common throughout the fungal kingdom. We also note the possibility of noncanonical PkaA phosphorylation site(s) in AflR. Biochemical studies suggest that Pka recognizes and phosphorylates the consensus sequences identified in AflR (Kemp and Pearson 1990) but noncanonical target residues have been described in other organisms including yeast (Pearson and Kemp 1991). It is possible that phosphorylation of noncanonical Pka motifs could account for some of the difference in *stcU* expression and ST production in the two AflR^{S323AS381AS382A} strains shown in Figure 3. These two possibilities (noncanonical sites and indirect regulation by Pka) are not mutually exclusive.

The intensity of the GFP in the AflR^{S323AS381AS382A} strain may suggest that AflR half-life is shortened when the protein is phosphorylated (Figure 4). Other studies have shown that phosphorylation can be a tag for protein degradation (Whitmarsh and Davis 2000). *Saccharomyces cerevisiae* Sic1 mutants lacking three cyclin-dependent kinase sites are not subject to ubiquitination degradation and remain stable *in vivo* (Verma *et al.* 1997). We have observed in another study (Yu *et al.* 1996) that *aflR* transcript is expressed longer than *stc* transcripts and now speculate that this could be due to progressive phosphorylation of AflR protein which could lead to degradation and/or inability to bind to *stc* promoters or otherwise activate their expression (Whitmarsh and Davis 2000). In the case of *S. cerevisiae* transcription factor ADR1, phosphorylation by Pka inhibits its ability to activate *ADH2* transcription but does not appear to affect its ability to bind to DNA (Taylor and Young 1990). Regardless of mechanism of decreased *stcU* transcription when AflR is phosphorylated, our results strongly suggest that AflR activity is negatively regulated by direct phosphorylation by PkaA.

Our GFP data also suggested that cellular machinery, under the control of PkaA, is involved in the localization of AflR to the nucleus (Figure 4). This would not be unprecedented as several studies of yeast transcription factors have demonstrated the involvement of Pka in nucleocytoplasmic shuttling of these proteins. Msn2 and Msn4 are *S. cerevisiae* transcription factors involved in the environmental stress response that are regulated by Pka activity. Evidence suggests that Pka phosphorylation of both of these factors is involved with their export from the nucleus (Görner *et al.* 1997). Pka activity is also associated with Rst2 localization. Rst2 is located nuclearly in a Δ*pka* strain and cytoplasmically in a constitutively activated Pka strain, although it is not possible to say if phosphorylation is associated with import or export functions (Higuchi *et al.* 2002). Mig1, involved in glucose repression in yeast, also changes locale coincident with changes in its phosphorylation status (De Vit *et al.* 1997). Although all of these yeast transcription factors are C2H2 zinc finger proteins and AflR is a Zn(II)2Cys6 binuclear cluster protein, it is likely cellular location of both protein classes is similarly regulated by a phosphorylation cascade. Another difference in our system is that the phosphorylation of AflR itself does not appear to play a sole role in localization; rather we hypothesize that an unknown factor(s) regulated by PkaA phosphorylation is essential for AflR import into the nucleus.

The experiments described here clearly demonstrate the importance of PkaA for AflR activity. Another major goal of this study was to determine if FlbA post-transcriptional regulation of AflR was mediated through PkaA. Our previous work indicated a role for PkaA in FlbA transcriptional regulation of AflR because deletion of *pkaA* restored *aflR* expression and subsequent ST production in the Δ*flbA* background (Shimizu and Keller 2001). We therefore thought it possible that an interaction of *pkaA* could explain why overexpression of *aflR* did not restore ST production in the Δ*flbA* background (Figure 5). However, contrary to our expectations, we found that *aflR*^{S323AS381AS382A} expression did not remediate *stcU* expression or ST production in the Δ*flbA* background. Furthermore, comparison of localization of the GFP::AflR fusion protein in the Δ*flbA* (*e.g.*, nucleus) and overexpression *pkaA* (*e.g.*, cytoplasm) strains indicated a different mechanism of AflR control by FlbA and PkaA. Thus, it appears AflR is regulated not only by PkaA but also by some unknown cellular component(s) dependent on FlbA activity. This situation is not unlike that suggested for Rst2 regulation, which has both Pka and non-Pka regulatory components, the latter possibly involved in environmental stimuli (Higuchi *et al.* 2002). To our knowledge, this is the first description of the requirement of an RGS protein for post-transcriptional regulation of a transcription factor in any system although recent work demonstrates a direct role of an RGS protein in transcriptional repression (Chatterjee and Fisher 2002) distinct from G-protein regulation.

In contrast to the interaction of FlbA and PkaA in transcriptional regulation of AflR and the apparent lack of this interaction in post-transcriptional regulation, we found that only RasA^{G17V} post-transcriptional regulation of AflR activity was partially mediated through PkaA (Figure 8). Deletion of *pkaA* did not restore ST biosynthesis in the RasA^{G17V} background but did partially restore *stcU* expression and ST production in the RasA^{G17V} background when *aflR* was overexpressed. This suggests an overlapping downstream target(s) of PkaA and RasA. One possible target could be LaeA, as we have found *laeA* transcription is repressed by both PkaA and RasA (J.-W. Bok and N. P. Keller, unpublished data). Overall though, it appears that RasA regulation of AflR is largely independent of PkaA. Examination of GFP was

useful in demonstrating a difference between RasA and PkaA regulation of AflR. GFP fluorescence was found in the nucleus in the *rasA*^{G17V}; OE::*gfp::aflR* strain (RTPH4.1) despite an inability of AflR to function; this was similar to the appearance of GFP in the Δ*flbA* strain. The nonfunctionality of nuclear AflR in the Δ*flbA* and *rasA*^{G17V} strains supports a possible convergence in AflR regulation by FlbA and RasA.

Perhaps the most significant finding in this study is the elucidation of elaborate cellular conduits controlling *aflR* gene expression, AflR activity, and, subsequently, ST production. This begs the question of why an organism would evolve such an intricate system to control secondary metabolite production. Although the biological effects of ST and AF are well documented, little data have suggested that these toxic and carcinogenic properties serve a function for Aspergillus spp. in nature. Recently, however, it has been shown that loss of *aflR* reduces fitness of *A. nidulans* as determined by a decrease in asexual spore production (Sim 2001; Ramaswamy 2002). This suggests that precise levels and activity of AflR might be critical for normal growth and development of the fungus and hence the requirement for such complex checks and balances in regulating its activity.

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