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fluG and *flbA* function interdependently to initiate conidiophore development in *Aspergillus nidulans* through *brlAβ* activation

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The *Aspergillus nidulans fluG* gene is necessary for the synthesis of a small diffusible factor that is required for the endogenously regulated induction of asexual sporulation that takes place during the development of an air-exposed colony. Previous work established that FluG is present at nearly constant levels throughout the *Aspergillus* life cycle, leading to the hypothesis that FluG factor is constitutively produced and development initiates after its concentration surpasses a fixed threshold. Here we show that overexpression of *fluG* can overcome the developmental block normally imposed on vegetative cells in submerged culture and leads to the formation of complex conidiophores that are remarkably similar to wild-type conidiophores made by air-exposed colonies. This *fluG*-induced sporulation requires the activities of other early developmental regulatory genes including, *flbA*, *flbB*, *flbC*, *flbD*, *flbE* and *brlA*. The requirement for *flbA* in *fluG*-induced sporulation is particularly interesting because overexpression of *flbA* can also induce sporulation in submerged culture and this *flbA* activity requires *fluG*. The interdependence of *fluG* and *flbA* activities suggests a close relationship between the products of these two genes in controlling conidiophore development. In addition to the endogenous sporulation signal provided by *fluG*, several environmental factors, including air exposure, carbon or nitrogen stress, and increased osmolarity, can influence developmental activation. We demonstrate that each of these signals requires the *brlAβ* gene, but not *brlAα*, to initiate conidiophore development. We present a model to account for the complex genetic and environmental controls leading to the activation of *brlAβ* and sporulation.

Keywords: asexual sporulation/conidiation/extracellular signaling/filamentous fungi/microbial development

Introduction

The filamentous fungus *Aspergillus nidulans* reproduces asexually by forming multicellular structures called conidiophores that are each composed of stalks, vesicles, sterigmata (metulae and phialides), and form multiple chains of spores called conidia (for a review, see Timberlake, 1990). Although the signals controlling the onset of conidiophore development are complex, under normal growth conditions the only environmental requirement for *A.nidulans* sporulation is exposure of the growing colony to air. Other controls determining the initiation of conidiation are apparently genetically determined and lead to the programed activation of sporulation at a specific time early in the life cycle of the expanding colony (Axelrod, 1972; Pastushok and Axelrod, 1976).

One critical gene that is specifically required at an early step in activating the sporulation pathway is *fluG* (Adams *et al.*, 1992; Lee and Adams, 1994a). Mutations in *fluG* lead to an inability to make the transition from vegetative growth to conidiophore development and result in the uncontrolled proliferation of undifferentiated aerial hyphae, giving the colony a fluffy or cotton-like appearance (Adams *et al.*, 1992). The developmental block observed in *fluG* mutants precedes activation of *brlA*, a gene encoding a transcription factor that has been shown to be required for activation of development (Adams *et al.*, 1988). One important aspect of *fluG* developmental control is that the requirement for *fluG* can be suppressed by growing a *fluG* mutant strain next to a wild-type strain (Lee and Adams, 1994a). Under these conditions, a strong band of conidiation can be observed at the interface between two colonies, suggesting that the wild-type strain provides a missing extracellular factor to the mutant strain, stimulating development. This phenotypic rescue of the *fluG* mutant strain occurs even if two colonies are separated by a dialysis membrane having a pore size of 6–8 kDa. These results have led to the hypothesis that *fluG* is required for the synthesis of a low-molecular-weight factor that initiates the major, programed pathway for conidiophore development, independent of nutrient status, as long as cells are exposed to air (Lee and Adams, 1994a). The *fluG* mutant phenotype can also be partially suppressed by growing the mutant colonies in suboptimal growth conditions, indicating that without the *fluG* signal, *A.nidulans* can induce conidiation in response to environmental cues (Adams *et al.*, 1992). This environmentally regulated response presumably also occurs in the wild-type, but is overwhelmed by the more prolific conidiation resulting from the *fluG*-dependent response.

At least five loci in addition to *fluG*, designated *flbA*, *flbB*, *flbC*, *flbD* and *flbE*, are individually required for the normally programed switch from hyphal growth to conidiophore development and *brlA* expression (Wieser *et al.*, 1994). As with wild-type strains, mutants resulting from loss of the *flb* genes extracellularly rescue the *fluG* mutant sporulation defect (Lee and Adams, 1994a). Thus, these genes are predicted to function downstream of *fluG* and their products are required for responding to the FluG conidiation signal. Of these genes, *flbA* is the best candidate to be part of a signal-transducing mechanism because the predicted FlbA sequence has 30% identity with the *Saccharomyces cerevisiae SST2* product (Lee and Adams,

1994b), which regulates the activity of the G protein-mediated signaling pathway for responding to mating pheromone (Dietzel and Kurjan, 1987b). The similarity between FlbA and SST2 may indicate that FlbA functions somewhat like SST2, and is involved in regulating a signal transduction pathway for responding to the FluG signal. Because inappropriate overexpression of *flbA* in vegetative cells caused *brlA* mRNA accumulation and resulted in the development of reduced conidiophores, we proposed that *flbA* has a positive role in activating development (Lee and Adams, 1994b).

flbB, *flbC*, *flbD* and *flbE* most likely function at the step downstream of *fluG* (and *flbA*) in activating sporulation because these mutants still produce the FluG extracellular conidiation factor and rescue the *fluG* mutant phenotype. Mutations in each of these genes result in fluffy colonies in which some conidiophores are produced following prolonged growth. Genetic analysis showed that *fluG* and *flbA* are both epistatic to *flbB*, *flbC*, *flbD* and *flbE* (Wieser *et al.*, 1994). Both *flbC* and *flbD* encode DNA binding proteins, indicating that they probably function as transcription factors in controlling development-specific gene activation (Wieser and Adams, 1995; J.W.Fondon and T.H.Adams, unpublished). Although *flbC* and *flbD* mRNAs are present throughout the *A.nidulans* life cycle, overexpression of *flbD* (and probably *flbC*) induces sporulation in a *brlA*-dependent manner. This raises the interesting possibility that the activities of FlbC and FlbD are regulated in response to the FluG signal.

In this paper, we show that overexpression of *fluG* in *A.nidulans* vegetative cells is sufficient to cause asexual development and results in the formation of relatively normal conidiophore structures. We also show that *fluG*-induced *A.nidulans* conidiophore development required *brlA* and other fluffy genes, including *flbA*, *flbB*, *flbC*, *flbD* and *flbE*. The requirement of *flbA* for *fluG*-induced development is interesting because *fluG* is also required for *flbA* to activate development. Finally, we show that for the complex *brlA* locus (Prade and Timberlake, 1993), the *brlA*β transcript, but not *brlA*α, is needed for *fluG*- or *flbA*-induced sporulation. Because overexpression of either *fluG* or *flbA* in a *brlA*α^+ *brlA*β^- strain resulted in accumulation of the *brlA*α message without causing development, BrlAα and BrlAβ are proposed to have distinct functions in early development.

Results

Overexpression of fluG is sufficient to cause A.nidulans conidiophore development and brlA expression

Because FluG protein is present in the cytoplasm at nearly constant levels, we proposed that initiation of development could be determined by the time required to reach a threshold level of FluG factor or by the availability of some other component needed for responding to this factor (Lee and Adams, 1994a). To test the hypothesis that the concentration of FluG factor determines developmental timing, we examined the effect of overexpressing *fluG* mRNA by constructing a strain (TBN57.8) with an extra copy of the *fluG* gene fused to the promoter for the *A.nidulans* catabolic alcohol dehydrogenase gene (*alcA*). *alcA* transcription is nutritionally regulated so that high levels of expression are induced when cells are grown in threonine as the sole carbon source, but this expression is repressed in the presence of glucose (Lockington *et al.*, 1985; Gwynne *et al.*, 1987). When the wild-type and TBN57.8 strains were grown on solid media containing threonine as the sole carbon source, the time required for the first conidiophore to be produced in the center of each developing colony was ~33 h, indicating that increased *fluG* expression did not affect the timing of sporulation in the presence of air (data not shown). However, as shown in Figure 1E, overexpression of *fluG* in vegetative hyphae grown in submerged culture was sufficient to cause development. Conidiophore vesicles and sterigmata were observed by 18 h after *fluG* induction, and complete conidiophores were observed by 24 h post-induction. These conidiophores are remarkably similar to those normally made by wild-type strains exposed to air (compare Figure 1D and E). Figure 2 shows that *brlA* mRNA had accumulated to easily detectable levels in TBN57.8 by the time of conidiophore vesicle formation. No conidiation and no *brlA* mRNA were detectable in the wild-type strain grown under the same conditions.

fluG and flbA function interdependently in causing conidiophore development

Overproduction of *flbA*, a second fluffy gene acting before *brlA*, was previously shown to cause vegetative hyphal cells grown in submerged culture to produce reduced conidiophore-like structures (Figure 1B; Lee and Adams, 1994b). Because *flbA* mutant colonies produce wild-type levels of FluG polypeptide and can rescue the *fluG* conidiation defect extracellularly, we propose that *flbA* functions downstream of *fluG*. However, the phenotype of a *fluG*$^-$ *flbA*$^-$ double mutant most closely resembles the *flbA*$^-$ single mutant, as expected if *flbA* functions before *fluG* (Wieser *et al.*, 1994). This could be explained if *flbA* has a role in regulating hyphal growth that is distinct from its proposed role in regulating the response to the *fluG*-directed sporulation signal. In an attempt to clarify the genetic interactions between *flbA* and *fluG*, we constructed a *flbA* deletion mutant strain containing the *alcA(p):fluG* fusion and a *fluG* deletion mutant strain containing the *alcA(p):flbA* fusion. As shown in Figure 1F, we found that overexpression of *fluG* in a *flbA* null mutant failed to induce conidiophore development or *brlA* expression (Figure 2) as expected if *fluG* functions before *flbA* and requires *flbA* to activate development. Overexpression of *flbA* in a *fluG* deletion mutant also failed to induce conidiation or *brlA* mRNA accumulation, as expected if *flbA* functions before *fluG* (Figures 1C and 2). Thus, we propose that *flbA* and *fluG* function interdependently in activating *brlA* mRNA accumulation, and inducing conidiophore development.

All flb genes are required for fluG to cause development in submerged culture

Besides *flbA* and *fluG*, at least four other fluffy genes called *flbB*, *flbC*, *flbD* and *flbE* are part of the *fluG* programed conidiation pathway (Wieser *et al.*, 1994). Mutations in any one of these genes result in a phenotype described as delayed conidiation because mutant colonies are initially fluffy, but begin to produce conidiophores in the center ~1–2 days after conidiophores appear in wild-type colonies. Double mutants that are *fluG*$^-$ and have

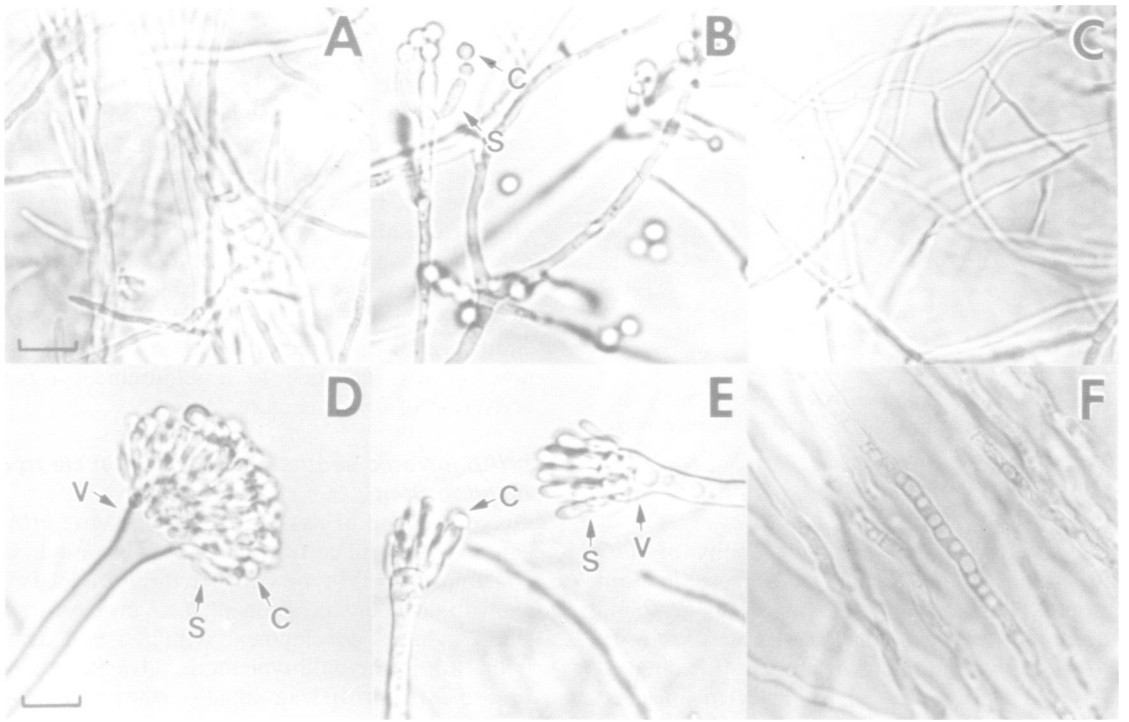

Fig. 1. Overexpression of *fluG* or *flbA* in vegetative cells causes conidiophore development. Strains TTA11 (**A**; wild-type), TBN54.10 (**B**; *alcA[p]::flbA*), RBN145 (**C**; *alcA[p]::flbA*; Δ*fluG*), TBN57.8 (**E**; *alcA[p]::fluG*) and RBN159 (**F**; *alcA[p]::fluG;* Δ*flbA*) were grown in *alcA(p)* repressing medium (glucose) for 14 h and then shifted to *alcA(p)*-inducing medium (threonine). Micrographs were taken 24 h after *alcA(p)*-induction. (**D**) A conidiophore produced by a wild-type strain during growth on the surface of a plate. V: vesicle; S: sterigmata; C: conidia. The scale bar in (D) is 10.6 μm and (B) and (E) have the same scale. The scale bar in (A) is 16 μm and (C) and (F) have the same scale.

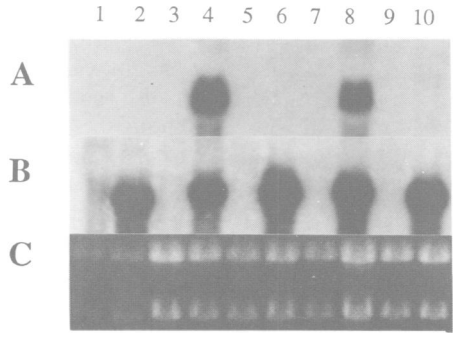

Fig. 2. Interdependence of *fluG* and *flbA* in activation of *brlA* expression. Total RNA (15 μg/lane) was isolated from a wild-type *A.nidulans* strain (lanes 1 and 2; TTA11), a strain containing the *alcA(p)::fluG* fusion (lanes 3 and 4; TBN57.8), a Δ*flbA* strain containing the *alcA(p)::fluG* fusion (lanes 5 and 6; RBN159), a strain containing the *alcA(p)::flbA* fusion (lanes 7 and 8; TBN54.10) and a Δ*fluG* strain containing the *alcA(p)::flbA* fusion (lanes 9 and 10; RBN145) and separated electrophoretically. Samples from lanes 1, 3, 5, 7 and 9 were taken from cultures grown under *alcA(p)*-repressing conditions (glucose medium) and samples from lanes 2, 4, 6, 8 and 10 were taken 24 h after shifting cultures to *alcA(p)*-inducing conditions (threonine medium). The blot in (**A**) was probed with a *brlA*-specific fragment and the blot in (**B**) was probed with a *alcA* coding region-specific fragment. Loading of total RNA was evaluated by ethidium bromide staining (**C**).

Table I. Effects of developmental mutations on the *fluG*-overexpression phenotype

Strain[a]	*brlA* expression	Sporulation	Vacuolation
RBN159 (Δ*flbA*)	–	–	+
RBN179 (*flbB*$^-$)	–	–	+
RBN157 (*flbC*$^-$)	–	–	–
RBN169 (*flbD*$^-$)	–	–	–
RBN178 (*flbE*$^-$)	–	–	–
RBN162 (*fluG*$^-$)	+	+	–
RBN195 (Δ*brlA*)	–	–	–
TBN57.8 (wild-type)	+	+	–
TTA11 (control)	–	–	–

Strains were grown as described in Materials and methods, and were observed at various times beginning 3 h post-induction and continuing through 24 h.
[a]All strains except TTA11 contain a copy of the *alcA(p):fluG* fusion.

delayed conidiation mutations are phenotypically *fluG* like, indicating that *fluG* is epistatic to *flbB*, *flbC*, *flbD* and *flbE*. To examine further the genetic requirements for *fluG*-induced sporulation in submerged culture, we have constructed *flbB*$^-$, *flbC*$^-$, *flbD*$^-$ and *flbE*$^-$ mutant strains containing the *alcA(p)::fluG* fusion. The strains were grown in glucose-containing medium (*alcA*-repressed condition) at 37°C for 14 h and shifted to threonine-containing medium (*alcA*-induced condition). All four genes were required for *fluG*-induced conidiation, as predicted if they function after *fluG* in controlling development (Table I). In addition, *brlA* mRNA did not accumulate in any of these mutants following *fluG* induction. For all of the strains except RBN179 (*alcA[p]::fluG; flbB*$^-$) *fluG* mRNA and protein levels did increase following transfer to *alcA*-inducing medium, indicating that the lack of development in these strains did not result from alteration of *alcA* promoter regulation (not shown). Several additonal *flbB*$^-$ *alcA(p)::fluG* strains were examined and in no case was *fluG* mRNA or protein detected (not shown). We consider it likely that the inability to detect increased *fluG* expres-

Table II. Effects of developmental mutations on the *flbA*-overexpression phenotype

Strain[a]	*brlA* expression	Sporulation	Vacuolation
RBN180 (*flbB*$^-$)	–	–	+
RBN151 (*flbC*$^-$)	+	+	+
RBN149 (*flbD*$^-$)	+	+	+
RBN155 (*flbE*$^-$)	+	+	+
RBN145 (Δ*fluG*)	–	–	+
RBN197 (Δ*brlA*)	–	–	+
TBN54.10 (wild-type)	+	+	+
TTA11 (control)	–	–	–

Strains were grown as described in Materials and methods, and were observed at various times begining 3 h post-induction and continuing through 24 h.
[a]All strains except TTA11 contain a copy of the *alcA(p)::flbA* fusion.

sion in these strains resulted from instability of *fluG* mRNA and protein in these mutants because induced expression of *fluG* in these *flbB*$^-$ strains had profound effects on growth and hyphal vacuolation (Table I). Moreover, *alcA* mRNA (not shown) and *alcA(p)*-directed *flbA* mRNA (Table II) were easily detected in *flbB*$^-$ mutants, supporting the hypothesis that the *alcA(p)* could be activated in a *flbB*$^-$ mutant.

Genetic requirements for flbA-induced sporulation

Double mutants that are *flbA*$^-$ and either *flbB*$^-$, *flbC*$^-$, *flbD*$^-$ or *flbE*$^-$ have a fluffy autolysis phenotype that is indistinguishable from *flbA*$^-$ single-mutant colonies, indicating that *flbA* is epistatic to *flbB*, *flbC*, *flbD* and *flbE* (Wieser *et al.*, 1994). To determine whether any of these genes are required for *flbA* overexpression to cause development, we constructed *alcA(p)::flbA* fusion strains having *flbB*$^-$, *flbC*$^-$, *flbD*$^-$ or *flbE*$^-$ mutations. As summarized in Table II, conidiophore development was observed and *brlA* mRNA was detected when *flbA* was overexpressed in *flbC*$^-$, *flbD*$^-$ and *flbE*$^-$ mutants. In contrast, *brlA*, *flbB* and *fluG* were each required for *flbA* to induce conidiophore development or *brlA* mRNA accumulation. *flbA* mRNA levels increased in all strains following transfer to *alcA(p)* inducing medium, indicating that the failure to develop was not due to a block in *alcA(p)* directed *flbA* induction (not shown).

brlAβ, but not brlAα, is required for fluG- or flbA-induced A.nidulans sporulation

brlA is a complex locus consisting of two overlapping transcripts called *brlA*α and *brlA*β. The predicted *brlA*β polypeptide has the same amino acid sequence as BrlAα, except that BrlAβ has 23 additional codons at its N-terminus (Prade and Timberlake, 1993). *brlA*β and *brlA*α are controlled by different mechanisms (Han *et al.*, 1993). *brlA*β expression is regulated at both the transcriptional and post-transcriptional levels, while *brlA*α expression is transcriptionally regulated and its expression is at least partially dependent on *brlA*β activity. Han *et al.* (1993) proposed that *brlA*β is required for the initiation of conidiophore development and this leads to activation of *brlA*α expression, maintaining conidiophore development. To determine whether overexpression of *flbA* or *fluG* activates conidiophore development through activation of *brlA*α or *brlA*β, we constructed *alcA(p)::flbA* and *alcA(p)::fluG* fusion strains that were *brlA*α$^+$ *brlA*β$^-$ or *brlA*α$^-$ *brlA*β$^+$. Figure 3 shows that overexpression of either *flbA* or *fluG* in the *brlA*α$^-$ *brlA*β$^+$ strains caused conidiophore development. By contrast, overexpression of either *fluG* or *flbA* in the *brlA*α$^+$ *brlA*β$^-$ strains failed to cause conidiation and resulted in a phenotype like that observed following *fluG* or *flbA* overexpression in a *brlA* deletion strain (Figure 3A, B, D and E). Surprisingly, *brlA*α mRNA was easily detectable by 6 h after inducing overexpression of either *fluG* or *flbA* in the *brlA*α$^+$ *brlA*β$^-$ strains, indicating that the failure of these strains to develop was not due to a requirement for *brlA*β in activation of *brlA*α (Figure 4).

brlAβ intronic sequences function in cis to delay development

Overexpression of *flbA* or *fluG* in a *brlA*α$^-$ *brlA*β$^+$ strain caused development faster than overexpression in a strain containing a wild-type copy of the *brlA* locus. Figure 5 shows that overexpression of *fluG* in a *brlA*α$^-$ *brlA*β$^+$ strain induced development within 6–9 h as compared with 18 h in the wild-type strain. Overexpression of *flbA* in a *brlA*α$^-$ *brlA*β$^+$ strain also caused development to occur faster than in a wild-type strain (by ~3 h, data not shown). These results indicate that the wild-type *brlA* locus has a negative effect on conidiophore development. The *brlA*α$^-$ *brlA*β$^+$ mutant locus differs from the wild-type *brlA* locus in that *brlA*β intron sequences have been deleted to eliminate the *brlA*α transcription start site. Thus, the faster development observed following overexpression of *fluG* or *flbA* in a *brlA*α$^-$ *brlA*β$^+$ strain could result from the absence of *brlA*α or removal of the *brlA*β intron. To distinguish between these possibilities, we constructed diploid strains containing the *alcA(p)::fluG* fusion and various combinations of *brlA* alleles. Table III shows that overexpression of *fluG* in all diploid strains containing the *brlA*α$^-$ *brlA*β$^+$ allele initiated conidiophore development more quickly than when *fluG* was overexpressed in a wild-type strain. This was even the case when *brlA*α$^-$ *brlA*β$^+$ was placed in *trans* to a wild-type *brlA* locus, indicating that the mutant locus is dominant. These results support the hypothesis that the *brlA*β intron contains *cis*-acting regulatory sequences that contribute to determining the timing of *brlA* activation and conidiation.

Requirements for fluG and flbA for stress-induced conidiation

Although *A.nidulans* conidiation does not normally take place during growth in submerged culture, it has been shown that wild-type *A.nidulans* will sporulate in liquid culture under special environmental conditions (Morton, 1961; Saxena and Sinha, 1973; Martinelli, 1976; Skromne *et al.*, 1995). Most recently, Skromne *et al.* (1995) showed that transferring wild-type *A.nidulans* hyphae from nutritionally complete liquid medium to liquid medium lacking either a carbon or nitrogen source resulted in conidiophore development and *brlA* activation. We found previously that *fluG* deletion mutants could be induced to conidiate when grown in the presence of air if growth was limited, suggesting that environmental stress-induced conidiation does not require *fluG*. To test whether *fluG* is required for sensing nutrient starvation when *A.nidulans* is grown in

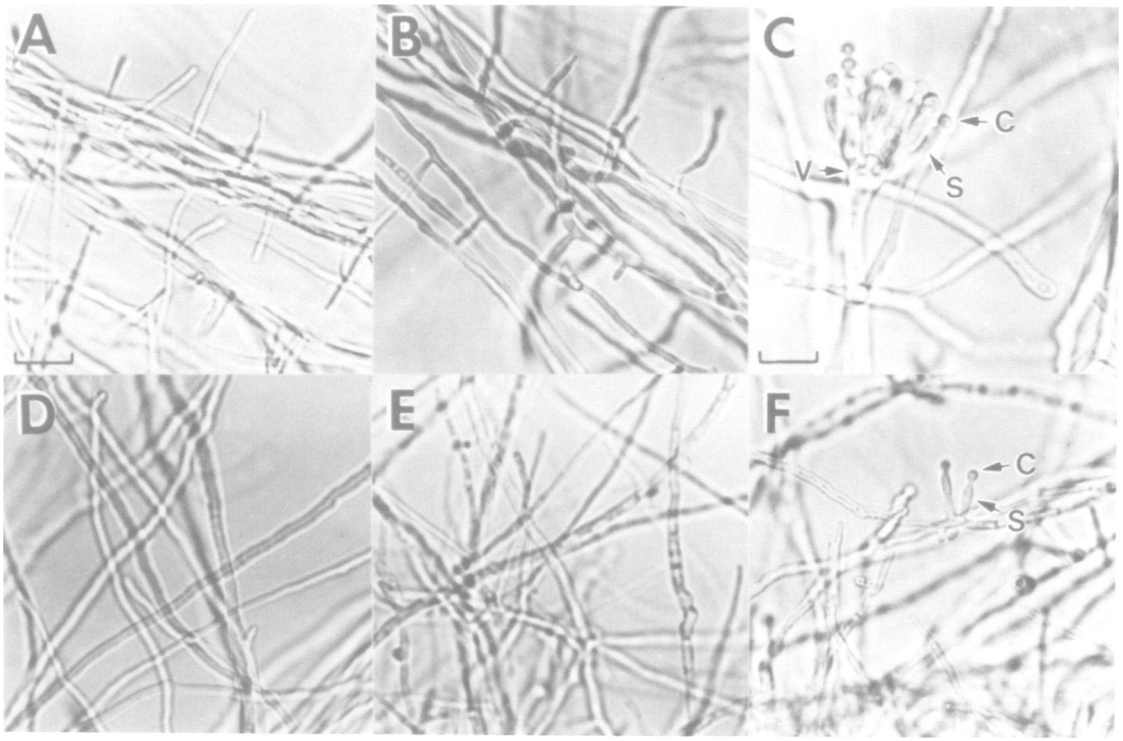

Fig. 3. *brlA*β, but not *brlA*α, is sufficient to cause conidiophore development. Strains RBN195 (**A**; *alcA[p]::fluG*, Δ*brlA*), RBN187 (**B**; *alcA[p]::fluG*, *brlA*α$^+$ *brlA*β$^-$), RBN184 (**C**; *alcA[p]::fluG*, *brlA*α$^-$ *brlA*β$^+$), RBN197 (**D**; *alcA[p]::flbA*, Δ*brlA*), RBN141 (**E**; *alcA[p]::flbA*, *brlA*α$^+$ *brlA*β$^-$) and RBN191 (**F**; *alcA[p]::flbA*, *brlA*α$^-$ *brlA*β$^+$) were grown for 14 h in *alcA(p)*-repressing medium and shifted into *alcA(p)*-inducing medium. Micrographs shown were taken at the 24 h post-induction time point, except (F) (6 h). The scale bar (A) is 10.6 μm.

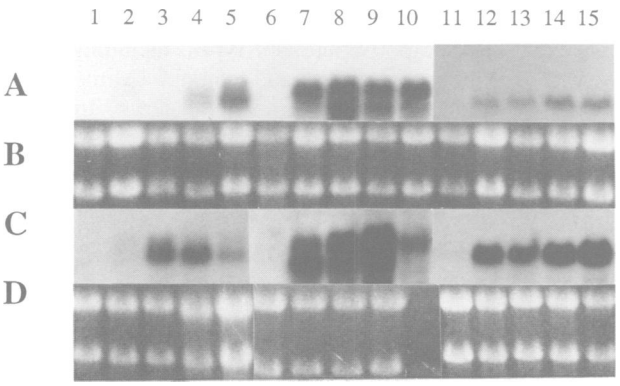

Fig. 4. Overexpression of *fluG* or *flbA* induces *brlA*α and *brlA*β mRNA accumulation. Total RNA (15 μg/ lane) was isolated from a wild-type strain containing an *alcA(p)::fluG* fusion [TBN57.8; (**A**) lanes 1–5], a *brlA*α$^-$ *brlA*β$^+$ strain containing the *alcA(p)::fluG* fusion [RBN184; (A) lanes 6–10], a *brlA*α$^+$ *brlA*β$^-$ strain containing the *alcA(p)::fluG* fusion [RBN187; (A) lanes 11–15], a wild-type strain containing *alcA(p)::flbA* fusion [TBN54.10; (**C**) lanes 1–5], a *brlA*α$^-$ *brlA*β$^+$ strain containing the *alcA(p)::flbA* fusion [RBN191; (C) lanes 6–10] and a *brlA*α$^+$ *brlA*β$^-$ strain containing *alcA(p)::flbA* fusion [RBN141; (C) lanes 11–15]. Strains were grown in for 14 h in *alcA(p)*-repressing conditions (lanes 1, 6 and 11) and then shifted to *alcA(p)*-inducing conditions and allowed to grow 6 h (lanes 2, 7 and 12), 9 h (lanes 3, 8 and 13), 12 h (lanes 4, 9 and 14) and 24 h (lanes 5, 10 and 15). The blots were probed with a *brlA*-specific fragment. Loading of total RNA was evaluated by ethidium bromide staining (**B** and **D**).

submerged culture, we grew the wild-type (TTA11) and *fluG* deletion (TTA127.4) strains for 18 h in standard minimal medium and then shifted to the same medium, but lacking either the carbon or nitrogen source. We found that *fluG* was required for responding to nitrogen starvation, but not carbon starvation (Figure 6, Table IV) indicating that carbon and nitrogen starvation induce *A.nidulans* conidiation through different mechanisms. Similar experiments using the *flbA* deletion mutant showed that *flbA* was required for responding to both nitrogen and carbon starvation (Table IV).

Previously, we showed that while several different environmental stresses partially rescued the *fluG* mutant phenotype, *flbA* mutants sporulated only when colonies were grown on high-osmolarity medium (Lee and Adams, 1994b). This led us to examine the possibility that increased osmolarity in *flbA* or *fluG* deletion mutants could cause sporulation. As described in Table IV, neither *fluG* nor *flbA* deletion mutant strains sporulated in submerged culture following transfer from standard minimal medium to the same medium containing 0.8 M sodium chloride, the condition that best suppresses the *flbA* mutant conidiation defect in air-exposed colonies. However, wild-type did produce conidiophores within 6–9 h of transfer to high-osmolarity medium. Thus, osmolarity can also overcome the need for air in inducing sporulation, but both *fluG* and *flbA* are required for this response. Finally, to test whether carbon stress, nitrogen stress and increased osmolarity induced sporulation in submerged culture through activation of *brlA*α or *brlA*β, RBN181 (*brlA*α$^+$ *brlA*β$^-$) and TJN5.23 (*brlA*α$^-$ *brlA*β$^+$) strains were grown in standard minimal medium and then shifted into either carbon-free, nitrogen-free or 0.8 M sodium chloride minimal medium. In each case, *brlA*β, but not *brlA*α, was found to be required to cause conidiophore development (Table IV).

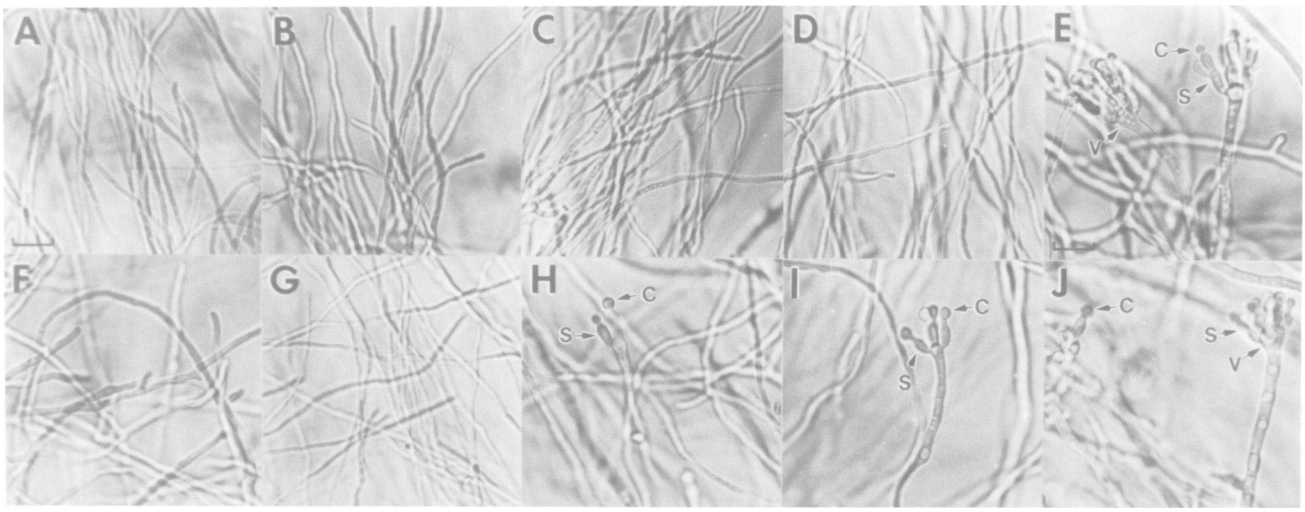

Fig. 5. Overexpression of *fluG* in *brlA*β-containing strains causes development faster than in wild-type. Strains TBN57.8 (**A–E**; *alcA[p]::fluG*) and RBN184 (**F–J**; *alcA[p]::fluG*, *brlA*α$^-$ *brlA*β$^+$) were grown for 14 h in *alcA(p)*-repressing medium and then shifted into *alcA(p)*-inducing medium. Cultures were observed and photographed at the time of the shift (0 h; A and F) and 6 h (B and G), 9 h (C and H), 12 h (D and I) and 24 h (E and J) after *alcA(p)* induction. The scale bar in (A) is 10.6 μm and all other panels are the same scale.

Table III. Dominance relationships for *brlA* mutant alleles

Strain[a]	Relevant genotype	Time development first observed (post-induction) (h)
DBN10[b]	$\frac{brlA\alpha^-\ brlA\beta^+}{brlA\alpha^-\ brlA\beta^+}$	6–9
DBN11[c]	$\frac{brlA\alpha\ brlA\beta^+}{\Delta brlA}$	6–9
DBN12[d]	$\frac{brlA\alpha^-\ brlA\beta^+}{brlA\alpha^+\ brlA\beta^-}$	6–9
DBN13[e]	$\frac{brlA\alpha^-\ brlA\beta^-}{brlA\alpha^+\ brlA\beta^-}$	6–9
RBN184	*brlA*α *brlA*β$^+$	6–9
TBN57.8	*brlA*α$^+$ *brlA*β$^-$	18–24

[a]All strains contain one copy of the *alcA(p)::fluG* fusion.
[b]Diploid isolated from heterokaryon between RBN184 and RBN175.
[c]Diploid isolated from heterokaryon between RBN184 and RSH94.4.
[d]Diploid isolated from heterokaryon between RBN184 and RBN181.
[e]Diploid isolated from heterokaryon between RBN184 and FGSC237.

Discussion

The normally regulated activation of conidiophore development in *A.nidulans* is dependent upon air and on a wild-type copy of the *fluG* gene (Adams *et al.*, 1992; Adams, 1994). This requirement for *fluG* can be suppressed extracellularly by wild-type strains, leading us to suggest that FluG functions in the production of a diffusible signal that determines initiation of the conidiation pathway (Lee and Adams, 1994a). Because FluG is present at nearly constant levels throughout the life cycle, even under conditions that do not support conidiation, we have speculated that FluG directs a low level of constitutive production of this sporulation-inducing factor with developmental induction occurring only once a threshold level of the factor accumulates (Lee and Adams, 1994a). The results presented in this paper demonstrate that overexpression of *fluG* in submerged culture is sufficient to overcome the usual requirement for air in inducing *A.nidulans* conidiophore development, indicating that FluG expression levels limit development in submerged culture. In contrast, the time required for conidiophore development to begin in an air-exposed colony was not altered by overexpressing *fluG*. Thus, while the lack of development observed in submerged cultures of *Aspergillus* results in part from a failure to accumulate sufficient FluG factor, developmental timing in surface colonies is apparently not determined solely by FluG factor levels.

The conidiophores produced following overexpression of *fluG* are remarkably similar to wild-type conidiophores and have all the appropriate cell types, including stalks, vesicles, metulae, phialides and conidia (Mims *et al.*, 1988). This contrasts with the results observed following overexpression of *flbA* or *brlA*, which both resulted in the production of relatively simple conidiophores (Adams *et al.*, 1988; Lee and Adams, 1994b). This result is consistent with the hypothesis that *fluG* functions as a very early regulator in the conidiation pathway and that activation of *fluG* results in activation of all the genes needed to make a complete conidiophore. Because strains with mutations in *flbA*, *flbB*, *flbC*, *flbD* or *flbE* are able to rescue the *fluG* mutant conidiation defect extracellularly, the products of each of these genes presumably function in responding to the FluG signal. As this model predicts, the *flb* genes are each required for *fluG* to induce sporulation in submerged culture, supporting the hypothesis that *fluG* encodes the signal producer and *flbA–flbE* encode signal regulators. We showed previously that *flbC* functions separately from *flbB*, *flbD* and *flbE*, and that *flbB* (but not *flbE*) was required for overexpression of *flbD* to induce development, leading to the proposed gene order presented in Figure 7 (Wieser *et al.*, 1994; Wieser and Adams, 1995). Here we showed that *flbB* is also distinguished from *flbD* and *flbE* in that overexpression of *fluG* in a *flbB* mutant (but not in *flbD* or *flbE* mutants) caused severe growth inhibition and extensive hyphal vacuolation (Table I). Understanding the basis for this differential response to *fluG* overexpression awaits the isolation and characterization of the wild-type *flbB* gene.

The interdependence of *fluG* and *flbA* in activating *brlA*

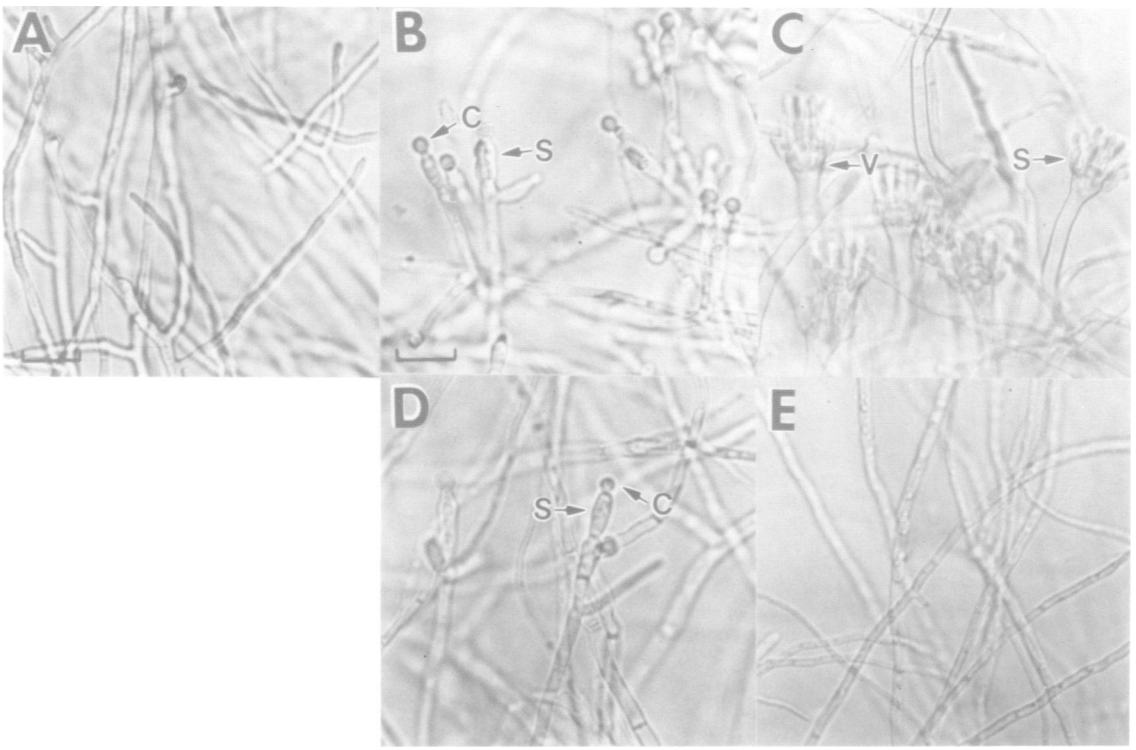

Fig. 6. *fluG* is required for nitrogen stress-induced conidiophore development. Wild-type (TTA11; **A–C**) and a *fluG* deletion mutant (TTA127.4; **D** and **E**) were grown in glucose minimal medium for 18 h (A) and then shifted to minimal medium lacking a carbon source (B and D) or minimal medium lacking a nitrogen source (C and E). Wild-type responded to carbon- or nitrogen-free medium by inducing conidiophore development within 12 h of the medium shift (B and C). The *fluG* deletion mutant responded to carbon stress by forming conidiophores within 12 h after the medium shift (D) but failed to respond to nitrogen stress, even by 24 h after shifting (E). The scale bar in (A) is 16 μm and (E) is the same scale. The scale bar in (B) is 10.6 μm and (C) and (D) are the same scale.

Table IV. Environmental signals inducing submerged conidiation

Strains	Growth medium[a]			
	Glucose, nitrate	No carbon, nitrate	Glucose, no nitrogen	Glucose, nitrate 0.8 M NaCl
TTA127.4 (*ΔfluG*)	–	+	–	–
TBN39.5 (*ΔflbA*)	–	–	–	–
TJN5.23 (*brlAα*$^-$, *brlAβ*$^+$)	–	+	+	+
RBN181 (*brlAα*$^+$, *brlAβ*$^-$)	–	–	–	–
TTA11 (wild-type)	–	+	+	+

[a]Cultures were grown in liquid minimal medium with glucose and nitrate for 18 h at 37°C, and then shifted to minimal medium containing the carbon and nitrogen sources indicated.
+ indicates that conidophore development and *brlA* mRNA accumulation could be detected by 12 h after transfer to these growth conditions.
– indicates that no conidiophore development or *brlA* mRNA accumulation could be detected even 24 h after shifting to these growth conditions.

expression and conidiophore development indicates a close relationship between *flbA* activity and the FluG signal. This result is consistent with our previous proposal that *flbA* functions in regulating the response to the FluG signal which was based primarily on the sequence similarity observed between *flbA* and *SST2*, a G-protein signaling regulator in the yeast mating pheromone pathway (Dietzel and Kurjan, 1987a; Lee and Adams, 1994b). However, it is important to point out that the genetic interactions between *flbA* and *fluG* are very complicated. Mutations in the *flbA* locus not only result in a fluffy phenotype, but also lead to the formation of interwoven hyphae that autolyse during colony aging. *flbA*$^-$ *fluG*$^-$ double mutants also have an autolytic phenotype, as expected if *flbA* functioned before *fluG*; however, this is inconsistent with the finding that *flbA* mutants still produce the Flug signal (Lee and Adams, 1994a; Wieser *et al.*, 1994). These contradictory results might be explained if *flbA* functions in regulating more than one signaling pathway: one controlling conidiophore development and a second controlling aspects of colony development. Along these lines, it is interesting that *flbA* (but not *fluG* or *flbD*) overexpression not only caused sporulation, but also resulted in increased expression of genes involved in the production of a polyketide secondary metabolite known as sterigmatocystin (Keller *et al.*, 1994; J.Hicks, N.Keller and T.H.Adams, unpublished). An alternative explanation for the interdependence of *flbA* and *fluG* in inducing conidiation results from a requirement for two independent routes to activate *brlA*. The coordinate activation of *brlA* by both pathways would be required to induce sporulation.

The interaction between *flbA* and other fluffy genes in

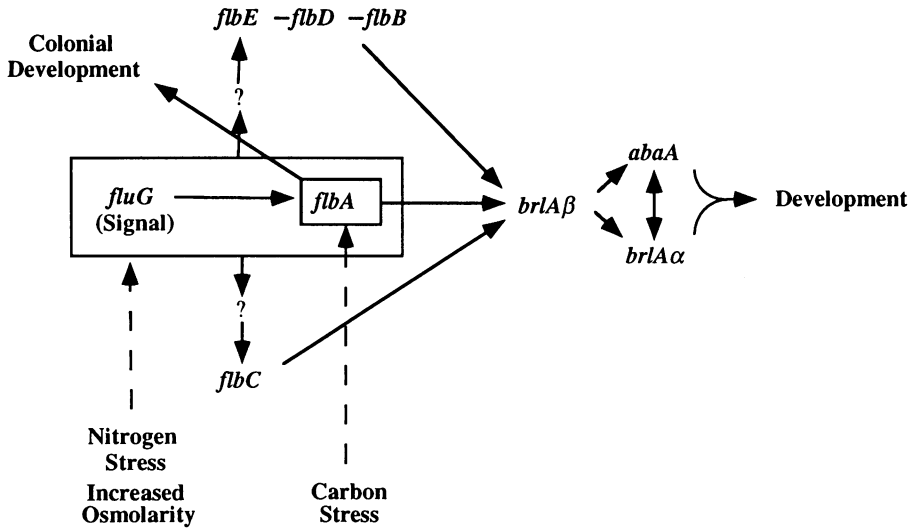

Fig. 7. Genetic model describing the control of developmental initiation in *Aspergillus*. We propose that *fluG* is responsible for producing a signal that functions extracellularly in controlling initiation of conidiophore development. *flbA*, *flbB*, *flbC*, *flbD* and *flbE* are required for responding to the FluG signal. *flbC* functions independently from *flbB*, *flbD* and *flbE* and the gene order *flbE* → *flbD* → *flbB* was shown by Wieser and Adams (1995). While *flbA* has a direct role in activating *brlA*, the position of *flbA* in this pathway is unclear. We have placed *flbA* downstream of *fluG* because *flbA* mutants can rescue the *fluG* mutant phenotype extracellularly. In addition, the similarity observed between *flbA* and yeast *SST2* supports the hypothesis that *flbA* functions in signal transduction. Because *flbA* mutations affect vegetative growth, we have proposed that *flbA* also controls other aspects of colony development that are independent of its role in conidiation. In addition to the endogenous *fluG* induction pathway, conidiation can also be activated by environmental cues, including carbon stress, nitrogen stress and increased osmolarity. These signals regulate development in *fluG*-dependent or *fluG*-independent ways, depending on the presence or absence of air. Finally, for each of the signals described, initiation of the conidiation pathway is dependent *brlA*β.

activating conidiation is also complicated by the fact that *flbA* may be involved in regulating additional pathways. We have proposed that *flbA* functions upstream of *flbB*, *flbC*, *flbD* and *flbE* primarily because mutations in these genes cause delayed conidiation, while *flbA* mutants are completely aconidial. Our results show that *flbB* is required for *flbA*-induced sporulation, as expected if *flbB* functions downstream of *flbA*. However, *flbC*, *flbD* and *flbE* are not required for *flbA* to induce development. We do not currently have a simple explanation for these results, but again point out the hypothesis that *flbA* controls more than one pathway. The genetic interactions among the products of these genes in activating *brlA* will require a more detailed understanding of how each gene functions.

The induction of sporulation by *fluG* or *flbA* overexpression requires the central regulatory gene, *brlA*. The *brlA* locus is complex, consisting of two overlapping transcripts designated *brlA*α and *brlA*β, both of which are required for normal conidiophore development (Han *et al.*, 1993; Prade and Timberlake, 1993). The BrlAα and BrlAβ polypeptides have been proposed to be functionally equivalent, but expression of the *brlA*β and *brlA*α genes is apparently controlled by different mechanisms (Han *et al.*, 1993). *brlA*β expression is regulated at both the transcriptional and post-transcriptional levels, while *brlA*α expression is transcriptionally regulated and its expression is at least partially dependent on *brlA*β activity. Han *et al.* (1993) proposed that activation of *brlA*β is required for initiation of conidiophore development. Activation of *brlA*β then activates *brlA*α and another downstream transcription factor *abaA* (Mirabito *et al.*, 1989; Andrianopoulos and Timberlake, 1994). AbaA both activates additional development-specific genes and feeds back to stimulate *brlA* expression to higher levels (Mirabito *et al.*, 1989). As predicted by this model, overexpression of either *fluG* or *flbA* in a *brlA*β$^+$ *brlA*α$^-$ strain was sufficient to cause conidiophore development, while no development was observed when either *fluG* or *flbA* was overexpressed in *brlA*α$^+$ *brlA*β$^-$ strains. Although no development took place following *fluG* or *flbA* overexpression in *brlA*α$^+$ *brlA*β$^-$ strains, *brlA*α mRNA accumulated to high levels, indicating that the failure to observe development in this mutant is not due to a lack of *brlA* activation, but may instead be due to a difference in BrlAα and BrlAβ activity. Because it is known that *brlA*α (or *brlA*β) overexpression by itself is sufficient to cause development (Adams *et al.*, 1988), it is apparent that there is a difference between *brlA*α activation in response to *fluG* and *flbA* overexpression, and direct *brlA*α activation (Adams *et al.*, 1988).

A surprising observation was that development resulting from *fluG* or *flbA* overexpression in the *brlA*α$^-$ *brlA*β$^+$ strain was significantly faster than in *brlA*α$^+$ *brlA*β$^+$ strains and resulted in the development of simpler conidiophore structures. This result was particularly striking in the *alcA(p)::fluG* fusion strain where the time required to produce conidiophore structures decreased from 18 h in the wild-type strain to 9 h in the *brlA*α$^-$ *brlA*β$^+$ strain. The primary difference between the *brlA*α$^-$ *brlA*β$^+$ mutant locus and the wild-type *brlA* locus is that the *brlA*β intron sequences were removed to eliminate the *brlA*α transcription start site. Thus, the consequence of this deletion is to simultaneously eliminate the *brlA*β intron and the *brlA*α transcript, either of which could result in faster development. By using diploid strains having various combinations of *brlA* mutant and wild-type loci, we found that deletion of the *brlA*β intron was dominant, indicating that the speedier developmental timing resulted from the removal of a negative acting element that functions *in cis*. It is not clear whether this change reflects a negative

Table V. *Aspergillus nidulans* strains

Strain	Genotype	Source
TBN57.8	*pabaA1, yA2; alcA(p)::fluG::trpC, trpC801, veA1*	this study
TBN54.10	*pabaA1, yA2; alcA(p)::flbA::trpC, trpC801, veA1*	this study
RBN21	*pabaA1, yA2; flbD14; veA1*	Wieser *et al.*, 1994
RBN26	*pabaA1, yA2; veA1; flbC8*	Wieser *et al.*, 1994
RBN70	*biA1; flbD14; trpC801, veA1*	Wiesser *et al.*, 1994
RBN138	*wA3; pyroA4; alcA(p)::flbA::trpC, veA1*	this study
RBN141	*pyroA4; brlAα⁻ brlAβ⁻, alcA(p)::flbA::trpC, veA1*	this study
RBN145	*pabaA1, yA2; fluG::argB; alcA(p)::flbA::trpC, veA1*	this study
RBN149	*pabaA1, yA2; flbD14; alcA(p)::flbA::trpC, veA1*	this study
RBN151	*pabaA1; wA3; alcA(p)::flbA::trpC, veA1; flbC8*	this study
RBN155	*pabaA1, yA2, flbE58; alcA(p)::flbA::trpC, veA1*	this study
RBN157	*pabaA1, yA2; alcA(p)::fluG::trpC, veA1; flbC8*	this study
RBN159	*pabaA1, ΔflbA::argB; alcA(p)::fluG::trpC, veA1*	this study
RBN162	*pabaA1, yA2; fluG::argB; alcA(p)::fluG::trpC, veA1*	this study
RBN195	*pabaA1; pyroA4; ΔbrlA::argB, alcA(p)::fluG::trpC, veA1*	this study
RBN169	*pabaA1, yA2; flbD14; alcA(p)::fluG::trpC, veA1*	this study
RBN175	*pabaA1, yA2; brlAα⁻ brlAβ⁺, veA1*	this study
RBN178	*pabaA1, yA2, flbE58; alcA::(p)fluG::trpC, veA1*	this study
RBN179	*yA2; flbB1; alcA(p)::fluG::trpC, veA1*	this study
RBN180	*biA1; flbB1, pyroA4; alcA(p)::flbA::trpC, veA1*	this study
RBN181	*pyroA4; brlAα⁻ brlAβ⁻, veA1*	this study
RBN184	*biA1; brlAα⁻ brlAβ⁻, alcA(p)::fluG::trpC, veA1*	this study
RBN187	*pryoA4; brlAα⁻ brlAβ⁻, alcA(p)::fluG::trpC,veA1*	this study
RBN191	*yA2; pyroA4; brlAα⁻ brlAβ⁺, alcA(p)::flbA::trpC, veA1*	this study
RBN197	*pyroA4; ΔbrlA::argB, alcA(p)::flbA::trpC,veA1*	this study
RJW58	*pabaA1, yA2, flbE58; veA1*	Wieser *et al.*, 1994
RJW120	*flbE58; methG1; veA1*	Wieser *et al.*, 1994
TBN39.5	*biA1, ΔflbA::argB; methG1; veA1*	Lee and Adams, 1994b
MBN001	*biA1; flbB1; veA1*	Wieser *et al.*, 1994
MBN008	*biA1; veA1; flbC8*	Wieser *et al.*, 1994
TFM5	*biA1; fluG::argB; methG1; veA1*	Adams *et al.*, 1992
TA046	*biA1; argB2; pyroA4; ΔbrlA::argB, veA1*	Han *et al.*, 1993
TTA11	*pabaA1, yA2; veA1*	Adams and Timberlake, 1990
TTA127.4	*pabaA1, yA2; ΔfluG::trpC; trpC801, veA1*	Lee and Adams, 1994a
TRP44L	*pabaA1, yA2; ΔargB::trpC; brlA44L (α⁻ β⁺)::argB, veA1*	Prade and Timberlake, 1993
FGSC237	*pabaA1, yA2; trpC801, veA1*	FGSC[a]
FGSC33	*biA1; pryoA4; veA1*	FGSC[a]
TJN5.23	*biA1; brlAα⁻ brlAβ⁺, veA1*	Jose Navarro
RSH94.4	*methG1; ΔbrlA, veA1*	Wieser and Adams, 1995

[a]Fungal Genetics Stock Center.

effect due to inefficient splicing of the intron or the presence of negative transcriptional regulatory elements encoded in the intron. In support of the second hypothesis, it has been previously suggested that *abaA* and *medA* can negatively affect *brlA* expression (Aguirre, 1993) and there are numerous AbaA binding sites within the *brlAβ* intron (Andrianopoulos and Timberlake, 1994). The simpler conidiophores produced in *brlAα⁻ brlAβ⁺* strains may indicate that the interactions of *brlAα* and *brlAβ* are necessary for the proper coordination of gene expression needed in forming wild-type conidiophores. Ultimately, the relative levels and timing of *brlAα* and *brlAβ* activation, as well as the spatial distribution of their products, must determine conidiophore morphology.

Finally, while *fluG* and *flbA* activate development through activation of *brlAβ*, several environmental inputs can contribute to activating *brlA* expression. This hypothesis is borne out by the fact that for air-exposed colonies the requirement for *fluG* in initiating development can be partially substituted by environmental cues such as nutrient deprivation, increased medium osmolarity and other conditions that inhibit growth. Moreover, Skromne *et al.* (1995) recently demonstrated that the need for air to initiate wild-type *A.nidulans* conidiation can be eliminated by severe nitrogen or carbon stress (nitrogen- or carbon-free medium) in liquid-grown culture. In addition, we have shown here that high osmolarity can bypass the need for air and cause *A.nidulans* to produce conidiophores in submerged culture. Somewhat surprisingly, *fluG* is required for nitrogen stress or increased osmolarity, but not carbon stress, to induce sporulation in submerged culture. In contrast to *fluG*, *flbA* was required for nutrient deprivation (carbon or nitrogen stress) and increased osmolarity to induce sporulation. These results could be interpreted to mean that both nitrogen and osmolarity signals function upstream of *fluG* when inducing submerged conidiation, whereas nitrogen, osmolarity and carbon function upstream of *flbA*. If this is the case, these stresses must regulate *flbA* and *fluG* activity in a post-transcriptional manner because none of these growth conditions caused an increase in *flbA* or *fluG* mRNA levels (not shown). This hypothesis also fails to explain why nitrogen stress and increased osmolarity bypass the need for *fluG*, and increased osmolarity bypasses the need for *flbA*, in air-exposed colonies. An alternative interpretation that fits both observations is that signals from *fluG*, nitrogen stress, osmolarity stress, air and carbon stress all have separate but additive functions in activating *brlA* expression and conidiation

initiation. Thus, when the combined activities of these different signals reach the appropriate level, development is initiated. In any case, nitrogen stress, carbon stress and high osmolarity all cause conidiation through the activation of *brlA*β, not *brlA*α, supporting the hypothesis that conidiophore development initiates through *brlA*β activation.

Materials and methods

Aspergillus strains, growth conditions and genetics

The haploid *A.nidulans* strains used in this study are described in Table V and diploid strains are described in Table III. Standard *A.nidulans* genetic (Pontecorvo *et al.*, 1953; Clutterbuck, 1974) and transformation techniques (Yelton *et al.*, 1984; Miller *et al.*, 1985) were used. All *A.nidulans* transformant strains used in this study were generated by integration of plasmid sequences at the *trpC* locus. Strains TBN54.10 and TBN57.8 containing the *alcA(p)* fused to *flbA* or *fluG* were constructed by transforming FGSC237 with pBN54 (Lee and Adams, 1994b) and pBN57, respectively. All developmental mutant strains containing *alcA(p)*gene fusions were constructed through meiotic crosses. Diploid strains containing the *alcA(p)::fluG* or *alcA(p)::flbA* constructs and heterozygous at *brlA* (Table III) were selected from heterokaryons of RBN184 and strains carrying appropriate *brlA* alleles. The presence of the *alcA(p)::fluG* or *alcA(p)::flbA* constructs and different *brlA* alleles in meiotic progeny or diploids was verified by Southern blot analysis.

In the *alcA(p)::flbA* or *alcA(p)::fluG* induction time course experiments, strains were inoculated at a density of 5×10^5 spores/ml in minimal nitrate medium (Käfer, 1977) containing 50 mM glucose and appropriate supplements, and shaken at 300 r.p.m. at 37°C for 14 h. Mycelia were harvested through MiraCloth (Calbiochem) washed once with minimal medium lacking glucose, and transferred to minimal medium containing 100 mM L-threonine and shaken for 24 h as above. Samples were taken at the time of the medium shift (0 h) and 3, 6, 9, 12, 18 and 24 h after the shift for observation and RNA isolation. For experiments that involved shifting strains to carbon- or nitrogen-free medium, 5×10^5 spores/ml were grown in minimal nitrate medium with glucose for 18 h and then shifted into the same minimal medium lacking either a carbon or a nitrogen source.

Nucleic acid isolation and manipulation

Total RNA was isolated as described previously (Adams *et al.*, 1988) and 15 μg/lane were separated by electrophoresis on formaldehyde–agarose gels and transferred directly onto nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL). Hybridization to ^{32}P-labeled random probes was according to procedures recommended by the membrane manufacturer. A 1.9 kb *Sal*I–*Hin*dIII fragment from plasmid pTA139 was used as a *brlA*-specific probe; a 2.5 kb *Xho*I fragment from plasmid pFM1 was used as a *fluG*-specific probe; a 3 kb *Eco*RI fragment from plasmid pBN30 was used as a *flbA*-specific probe; plasmid pJA1 was used as an *alcA*-specific probe.

The *alcA(p)::fluG* fusion vector PBN57.8 was constructed by first amplifying a 3 kb *fluG* fragment using genomic clone pFM1 (Adams *et al.*, 1992) as a template and the oligonucleotides AGATAGGATCCCGGCCTT and AGGAGAAAGCTTAGACTC as primers for the polymerase chain reaction (PCR). The resultant fragment was digested with *Bam*HI and *Hin*dIII, and inserted into the same sites in pBluescript-II KS(–) to give pBN56. The *fluG Bam*HI–*Hin*dIII fragment from pBN56 was then moved into pBN55 to give pBN57. pBN55 was made by transferring a 1.8 kb *Eco*RI–*Sst*I 5′-*trpC* fragment from pEP4 into PSH51 which is a pK19 vector containing the *alcA* promoter (Han *et al.*, 1993).

Microscopy

Photomicrographs of hyphal development were taken using an Olympus BH2 compound microscope and differential interference contrast optics.

Acknowledgements

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