Interaction between developmental and cell cycle regulators is required for morphogenesis in *Aspergillus nidulans*

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In *Aspergillus nidulans*, mutation of the transcriptional regulator *brlA* arrests formation of asexual spore-forming structures called conidiophores but does not hinder vegetative hyphal growth. During conidiophore development a 6-fold, *brlA*-dependent increase in the kinase activities of NIMX\(^{cdc2}\) and NIMA occurs. A similar level of kinase induction was promoted by ectopic expression of *brlA*. Northern and Western analysis revealed marked induction of *nimX^{cdc2}* mRNA after ectopic expression of *brlA* and increased amounts of NIMX\(^{cdc2}\). Therefore, *nimX^{cdc2}* is developmentally regulated by *brlA* indicating a direct role for *brlA* in the regulation of cell cycle genes. That correct regulation of *nimX^{cdc2}* is important for normal development was further supported by analysis of conidiophore development and septation in cell cycle specific mutants. Most noticeably, the *nimX^{cdc2AF}* mutation promoted inappropriate septation and hindered the switch from filamentous growth to budding growth seen during conidiophore development. Therefore, in contrast to the situation previously reported for other multicellular eukaryotes, interaction between developmental regulators and cell cycle regulators is essential for normal morphogenesis in *A.nidulans*.

Keywords: *brlA*/cell cycle/development/nimA/nimX\(^{cdc2}\)

Introduction

Developmental elaboration of multicellular organelles and tissues involves regulated growth, cell cycle progression and cytokinesis. How growth, cell cycle progression and cytokinesis are coordinately regulated during development is an active area of research but remains largely a mystery at the present time. Studies from both plant systems and *Drosophila* indicate that there may only be a weak link between cell cycle progression and morphogenesis, as experimental manipulation of rates of cell division has little effect on the timing or pattern of morphogenesis (Edgar and O’Farrell, 1989, 1990; Hemerly et al., 1995; Doerner et al., 1996).

Several aspects of the filamentous fungus *Aspergillus nidulans* make it an attractive organism in which to study the relationship between cell cycle and developmental controls (Mirabito and Osmani, 1994). Extensive research has defined networks of regulatory genes and their encoded proteins that control cell cycle progression or morphogenesis in this model filamentous fungus (Timberlake, 1990; Champe and Simon, 1992; Doonan, 1992; Adams, 1994; Osmani and Ye, 1996). The molecular tools are therefore available to ask questions experimentally about interactions between cell cycle control and morphogenesis. Additionally, *A.nidulans* elaborates several different cell types during asexual growth and spore development and the number of nuclei within each different cell type is specific, ranging from one nucleus per cell to >16 per cell (Mirabito and Osmani, 1994). These cells are elaborated using both filamentous and budding modes of growth. Because its modes of division are developmentally regulated, and its cells contain varying numbers of nuclei, the necessity for significant interaction between the developmental program of *A.nidulans* and cell cycle controls appears to be high (Mirabito and Osmani, 1994).

In this work, direct experimental data demonstrate that a high level of communication between developmental genes and cell cycle control genes exists in *A.nidulans* and that cell cycle control defects markedly affect morphogenesis.

Results

*p34^{cdc2}* and NIMA kinase activities are increased during conidiophore development in a *brlA*-dependent manner

*Aspergillus nidulans* cells growing in liquid culture (mycelia) do not produce asexual spores due to lack of an air–solid interface, although they are developmentally competent (Champe and Simon, 1992). Upon exposure to an air surface, mycelia differentiate, and aerial stalks crowned by vesicles are formed to a defined height. From the vesicle a series of cells, called metulae, are formed by budding. From metulae a second tier of cells called phialides are formed, which then continue to bud off round uninucleate spores (conidiospores) by repeated budding coupled to mitotic divisions. The entire spore-forming structure is known as a conidiophore (Timberlake, 1990; see also Figure 6D).

It is possible to induce conidiophore development synchronously by transferring a thin mycelial mat filtered from liquid culture to an agar plate (Boylan et al., 1987). At ~10 h conidiophore development is evident in a wild-type strain, while in *brlA* mutants aerial stalks form
Development and cell cycle in *Aspergillus nidulans*

**Fig. 1.** P34cdc2 (NIMXcdc2) and NIMA kinase activities are increased during conidiophore development in a *brlA*-dependent manner. Conidiophore development was induced by transferring liquid-grown cells of a wild-type strain and a strain containing a temperature-sensitive allele of *brlA* (A583) to agar plates incubated at 42°C to inactivate brlA. Both NIMA and p34cdc2 kinase activities were assayed from samples removed during conidiophore development at the times indicated. All activities are compared with that present in the liquid-grown cells before transfer to plates.

Normally but no further conidiophore development takes place (Adams *et al*., 1988). As shown in Figure 1, formation of conidiophores correlates with a significant increase in the activities of both p34cdc2 and NIMA kinases, which are both required for entry into mitosis in *A. nidulans* (Osmani and Ye, 1996). In contrast, a *brlA* mutant strain that failed to form conidiophores displayed no such increase in these kinase activities when treated in an identical manner (Figure 1). The activation of p34cdc2 and NIMA kinase activities is therefore associated with conidiophore development and requires the function of *brlA*.

To help study how *brlA* regulates p34cdc2 and NIMA kinase activities during conidiophore development, we analyzed the effect of unscheduled expression of *brlA* in liquid culture on these two kinases. The *brlA* gene is normally not expressed when *A. nidulans* is grown in liquid culture. However, expression of *brlA* from the highly regulated *alcA* promoter is able to induce conidial development in liquid culture (Adams *et al*., 1988). Transcription from the *alcA* promoter can be induced by ethanol and repressed by acetate. A *brlA* induction experiment was carried out as follows. *Aspergillus* cells were first grown overnight in acetate (repressing medium) to early log phase before transfer to ethanol (inducing medium). As previously described (Adams *et al*., 1988), expression of *brlA* in vegetative cells is sufficient to promote conidial development. After 4 h growth in inducing medium, numerous conidia were produced by budding division from both hyphal tips and subapical compartments, which were typically adjacent to a septum (data not shown). However, normal conidiophores with multiple specialized cell types were not induced by *brlA*. Instead, conidia were directly derived from hyphae by budding. These conidia were uninucleate as revealed by DAPI staining, and were morphologically very similar to conidia derived from naturally developed conidiophores (data not shown). In cells not expressing *brlA* such morphogenic changes were not observed and the cells continued polarized growth after a transient adaptation to the ethanol-containing medium.

Both p34cdc2 and NIMA kinase activities were markedly increased by 2–4 h following induction of *brlA* expression (Figure 2A). By 6 h the kinase activities declined as cells ceased growth in the presence of high levels of *brlA* (Adams and Timberlake, 1990). In contrast, in the absence of *brlA* expression, the kinase activities initially dropped after transfer to the inducing medium and then recovered by 4 h to their original levels (Figure 2A). Therefore, expression of *brlA* in vegetative mycelia is sufficient to activate p34cdc2 and NIMA kinases, supporting a close
link between activation of these cell cycle regulating kinases and brlA-mediated conidiophore development.

The expression of abaA is also required for conidial development and is under the control of brlA (Mirabito et al., 1989). To determine if abaA function is required for brlA-mediated activation of p34cdc2 and NIMA kinases we investigated whether abaA expression from the alcA promoter activates these kinases. As shown in Figure 2B, both p34cdc2 and NIMA kinase activities were significantly increased after induction of abaA expression. However, unlike brlA expression, which transiently enhances the kinase activities with peak activities usually at 2 h after induction, expression of abaA causes a gradual and continuous increase in the kinase activities (Figure 2B). As expression of abaA from the alcA promoter activates brlA expression by feedback regulation (Mirabito et al., 1989), it is possible that the activation of the two kinases by abaA expression may still be mediated via brlA. This would be consistent with the more gradual rise in the kinase activities observed in cells expressing abaA than in cells expressing brlA. We therefore expressed brlA from the alcA promoter in abaA-null cells and analyzed the consequence of its expression on p34cdc2 and NIMA kinase activities. Compared with that shown in Figure 2A, brlA expression transiently elevated p34cdc2 and NIMA kinase activities in the presence or absence of abaA. The data indicate that the brlA-mediated activation of both p34cdc2 and NIMA kinases is largely independent of abaA.

**Regulation of cell cycle mRNAs by brlA**

brlA encodes a transcriptional regulator containing two TFIIB-type zinc fingers (Adams et al., 1988). We therefore determined by Northern blot analysis whether brlA increases p34cdc2 and NIMA activity through enhanced expression of their mRNA. As shown previously, expression of brlA and abaA is absent in the vegetative hyphae (Figure 3A). Upon transfer to inducing media, brlA mRNA was transcribed from the alcA promoter and, as expected, subsequently induced the expression of abaA mRNA. Although the expression of nimXcdc2 and nimA could be detected in vegetative hyphae their expression was enhanced upon brlA expression (Figure 3A), particularly for nimXcdc2 mRNA. The enhanced expression of these key cell cycle regulators is not caused by the medium change as their expression levels did not change in the wild type. It is also unlikely that the enhanced expression is a general non-specific response to brlA overexpression as the level of 3’ phosphoglycerate kinase (PGK) did not increase but instead decreased after brlA induction (Figure 3A). In addition, the level of benA (β-tubulin) in these samples did not show increases either (data not shown). We conclude that the brlA developmental regulator increases p34cdc2 and NIMA kinase activity during conidiophore development through specific upregulation of nimXcdc2 and nimA gene expression, perhaps to coordinate the cell cycle with cell differentiation and morphogenesis of the conidiophore.

We also detected an increase in nimEcyclinB mRNA upon induction of brlA (Figure 4). At 1 h of induction an increase in full-length nimEcyclinB was observed (termed nimEα) but later a smaller species was induced to even higher levels. This smaller nimEcyclinB mRNA (termed nimEβ) is unlikely to be the result of RNA degradation as no such shifts were observed for several other mRNAs that were detected on the same blot. The induced expression of nimEβ is clearer using Southern blot analysis of double-stranded cDNA synthesized from poly(A)+ RNA (Figure 4B). It is possible that nimEα and nimEβ are derived from a different transcription initiation site. Such alternative initiation of overlapping transcription units has been found in two other developmentally regulated genes, brlA (Han et al., 1993; Prade and Timberlake, 1993) and slaA (Wu and Miller, 1998).

To see whether the smaller transcripts of nimEcyclinB are translated we compared protein levels of NimXcdc2 and NimEcyclinB by Western blotting before and after brlA induction. Similar to the transient activation pattern of p34cdc2 kinase (Figure 2), a transient increase in NimXcdc2 level was also detected at 2 and 4 h after brlA induction (Figure 3B). In contrast, NimEcyclinB levels remained essentially constant before dropping to a level barely...
to contain consensus sequence motifs designated brlA as response elements (BREs) (Chang and Timberlake, 1992). Sequences of genes regulated by conidiophore development. The upstream regulatory region contains multiple brlA response elements. We have found that the elevated p34 cdc2 kinase activity induced by brlA is likely to be due to higher levels of NIMX cdc2 rather than elevated NIME cyclinB.

To characterize further the nature of the smaller NIME cyclinB mRNA species we defined it using three different approaches (PCR-select cDNA subtraction differential screening, 5' and 3' RACE, and primer extension analysis). Results of these analyses collectively indicate that nime cyclinB mRNA is 77 bp shorter at its 5' end and 591 bp shorter at its 3' end. However, the protein coding capacities of nime cyclinB and nime cyclinBβ were found to be identical.

**The nimXcdc2 upstream regulatory region contains multiple brlA response elements**

brlA encodes a transcriptional activator that has been shown to promote transcription of numerous developmentally regulated genes, such as abaA and rodA, during conidiophore development. The upstream regulatory sequences of genes regulated by brlA have been found to contain consensus sequence motifs designated brlA response elements (BREs) (Chang and Timberlake, 1992). As nimXcdc2 expression appears to be directly regulated in response to the levels of brlA, we sequenced 1895 bp of putative upstream regulatory sequences of nimXcdc2 present in plasmid pNIG4 (Osmani et al., 1994) and searched for BREs (CAAGGG and C/A G/A AGGG G/A). We also searched for abaA response elements (AREs; CATT C/T) (Andrianopoulos and Timberlake, 1994), as nimXcdc2 is also responsive to this transcriptional activator. As BREs have been identified +150 from the transcription start site (Andrianopoulos and Timberlake, 1994) we included a further 150 bp present at the 5' end of nimXcdc2 cDNA in the search for BREs and AREs, making a total of 2045 bp. Three CAAGGG sequences were identified (at 284–279, 1932–1937 and 1963–1970) and four C/A G/A AGGG G/A sequences (at 209–204, 870–864, 1093–1099 and 1702–1708). In addition, two CATT C/T sequences were identified at 242–247 and 294–289. By randomizing the 2045 bp of nimXcdc2 genomic DNA 1000 times and re-searching for BREs we determined that the probability of getting seven BREs at random would be 0.017. However, identification of two AREs was not statistically significant. The presence of seven BREs therefore indicates that nimXcdc2 could be developmentally regulated directly by brlA-specific transcriptional activation.

**Regulation of p34cdc2 by tyrosine phosphorylation is required for correct cell pattern formation and suppression of septation during conidiophore development**

We recently generated the nimXcdc2AF mutant strain to study the role of tyrosine phosphorylation of p34cdc2 in cell cycle regulation (Ye et al., 1996). This strain can not negatively regulate p34cdc2 by threonine 14 or tyrosine 15 phosphorylation as these phosphorylation sites are mutated in the cdc2AF mutant strain to undergo development. However, tyrosine phosphorylation of p34cdc2 is required for correct cell pattern formation and suppression of septation during conidiophore development. To determine how non-tyrosine phosphorylated p34cdc2 affects conidiation, we followed...
Tyrosine phosphorylation of p34cdc2 is required for correct conidiophore morphogenesis. A wild-type strain (A and D) and a nimXcdc2AF strain (FRY-20-1) (B, C and E) were induced to undergo conidiation. Light and scanning electron microscopy were used to study morphogenesis of developing conidiophores. (A) and (B) have been stained with both DAPI and calcofluor. In (A) only a few nuclei are present in the vesicle as many have moved into the developing metulae but cannot be seen due to the intense calcofluor staining. (A) and (B) are fluorescence micrographs, (C) a phase contrast light micrograph, and (D) and (E) scanning electron micrographs. Bars, 10 µM; (A), (B) and (C) are at the same magnification.

conidiophore development in wild-type and the nimXcdc2AF mutant strains by light and scanning electron microscopy.

Normal conidiophores are highly symmetrical and consist of a stalk ending in a vesicle that is crowned by tiers of specialized metulae and phialides cells. Staining of conidiophores with DAPI to reveal nuclei, and calcofluor to stain septa, demonstrates that very few septa are seen in mature conidiophores and that the metulae and phialides stain intensely with calcofluor in a wild-type strain (Figure 6A). In contrast, mutant metulae and phialides apparently stained less intensely with calcofluor (Figure 6B). Additionally, in the nimXcdc2AF conidiophores many septa were apparent (Figure 6B, examples arrowed), separating nuclei into different compartments within the stalk and vesicle. Another distinctive feature of the nimXcdc2AF conidiophores is their lack of symmetry (Figure 6B, C and E) and their inability to develop the correct type of cell sequentially. For instance, normally a single vesicle is formed followed by a single tier of metulae and a single tier of phialides (Figure 6A and D). In the nimXcdc2AF mutant strain, many developing conidiophores form multiple vesicles. For example, in Figure 6C, at the apex of the original vesicle, rather than a tier of metulae cells, two additional vesicles have subsequently formed (arrowed).

Quantitation of the developmental defects indicate that no wild-type conidiophore heads are asymmetric, whereas 39.5% of the nimXcdc2AF strain’s conidiophores are markedly asymmetric. No wild-type conidiophores had multiple heads but 21.5% of the mutant conidiophores had multiple heads where the conidiophore was reiterated (e.g. Figure 6C). Finally, only 4.3% of wild-type conidiophores had a septum but 100% of the nimXcdc2AF mutant conidiophores contained multiple septa. Several different strains that had defects in tyrosine phosphorylation of p34cdc2 also displayed marked defects in conidiophore production and conidiophore differentiation and formed septa in the conidiophore stalk (data not shown). The data indicate that inability to tyrosine phosphorylate p34cdc2 interferes with the capacity of A.nidulans to change cell fate during asexual differentiation.

Discussion

Although much is known about cell cycle and developmental regulation in A.nidulans, little work has been
presented investigating the interaction between these two regulatory systems. During the development of asexual spores *A.nidulans* undergoes dramatic changes in its mode of growth and the degree of linkage between cytokinesis and nuclear division. We are interested in understanding how changes in the mode of growth, from hyphal to budding, are integrated with regulation of the cell cycle to form different cells containing from 1 to >16 nuclei per cell. The current work demonstrates that there is a high level of communication between cell cycle regulation and development and that *nimX<sup>cdc2</sup>* plays a pivotal role at this regulatory interface.

Two lines of evidence implicate *nimX<sup>cdc2</sup>* function at a focal point between regulation of the cell cycle and morphogenesis. First, its kinase activity is highly induced during the transition from indeterminate hyphal growth to differentiation of the specific cell types elaborated during conidiophore formation. This induction is dependent upon the *brlA*-encoded, master transcriptional regulator and could be inappropriately induced upon ectopic induction of *brlA*. *brlA* is therefore necessary and sufficient for increased *nimX<sup>cdc2</sup>* mRNA induction during asexual development. The upstream regulatory domain of *nimX<sup>cdc2</sup>* contains BRES, and induction of *brlA* leads to increases in the expression of both *nimX<sup>cdc2</sup>* mRNA and protein. This suggests that part of the function of *brlA* during conidiophore development is to regulate positively the amount, and subsequently the activity, of NIMX<sup>cdc2</sup>. Other cell cycle regulators were also found to be enhanced in a *brlA*-dependent manner and this may reflect a requirement for a higher nuclear proliferative capacity during rapid spore formation to generate the nuclei necessary for viable spore production.

Interestingly, a smaller *nimE<sup>cyclinB</sup>* mRNA species, termed *nimE<sup>B</sup>* was produced upon ectopic induction of *brlA*. This smaller mRNA is also induced during normal conidiophore development (B. Miller, personal communication). Although *brlA* induced high levels of both *nimE<sup>x</sup>* and *nimE<sup>B</sup>* mRNA no significant increase in the abundance of *nimE<sup>cyclinB</sup>* protein could be detected. Most noticeably, large amounts of *nimE<sup>B</sup>* failed to produce elevated levels of NIME. As the size of the *nimE<sup>B</sup>* transcript was significantly different to *nimE<sup>x</sup>* we considered that these two species may produce significantly different proteins. This was found not to be the case. However, *nimE<sup>B</sup>* has a different transcription start site reducing the 5' untranslated region from 642 to 51 bp. It also has a different polyadenylation site, which shortens the 3' untranslated region by 77 to 201 bp. As the coding capacities of *nimE<sup>x</sup>* and *nimE<sup>B</sup>* remain unchanged, perhaps the 5' and 3' untranslated regions of *nimE* mRNA play a regulatory role in the distribution of NIME mRNA, which could be important for correct localization of NIME within the conidiophore.

The second line of evidence implicating *nimX<sup>cdc2</sup>* in asexual development is the marked effect that the *nimX<sup>cdc2AF</sup>* mutation had upon septation and cell pattern formation during conidiophore development. This mutation makes NIMX<sup>cdc2</sup> resistant to negative regulation by *weel*-like tyrosine protein kinases, such as *ankA* in *A.nidulans* (Ye *et al.*, 1996, 1997). Lack of this negative regulation does not prevent cell cycle progression but does result in some loss of checkpoint regulation over mitosis and markedly impairs conidia production. During normal growth of the conidiophore, septation is repressed in the developing stalk but the cell cycle continues producing a large multinucleate cell that will subsequently populate metulae with nuclei as they are formed by budding from the apex of the vesicle. However, in the *nimX<sup>cdc2AF</sup>* conidiophores, septation is not uncoupled from nuclear division and multiple septa are formed in the developing stalk. This strongly indicates that septation is normally prevented in the developing conidiophore via a mechanism involving tyrosine phosphorylation of *nimX<sup>cdc2</sup>*.

Septation has previously been shown to be regulated by the DNA damage checkpoint via tyrosine phosphorylation of *nimX<sup>cdc2</sup>* (Harris and Kraus, 1998) in a manner analogous to how DNA damage prevents mitosis (Ye *et al.*, 1997). However, during conidiation, septation is not being modulated by external influences (such as DNA damage) but is modulated by the developmental program itself. Perhaps *brlA* promotes expression of negative regulators of *nimX<sup>cdc2</sup>* specific for its proposed septation-promoting functions. These factors would act to inhibit the ability of *nimX<sup>cdc2</sup>* to promote septation in the developing conidiophore but would be inactive against the *nimX<sup>cdc2AF</sup>* version of *nimX<sup>cdc2</sup>* allowing septation to proceed uninhibited.

In addition to allowing septation in vesicles the *nimX<sup>cdc2AF</sup>* mutation markedly modified the symmetry of the conidiophore and interfered with the normal progression of cell types elaborated during differentiation. Previous studies have demonstrated that impairing the function of NIMX<sup>cdc2</sup> to slow the rate of nuclear division secondarily impairs conidiophore development by preventing the normal progression of cell type formation (O’Connell *et al.*, 1992). As the *nimX<sup>cdc2AF</sup>* mutation has the opposite effect on the regulation of the cell cycle, allowing it to run without some of its normal constraints, lack of nuclear division is unlikely to be the cause of the developmental defects observed in this strain.

Nuclear positioning has been proven to be important for development of the conidiophore. Nuclear distribution mutants (*nud* mutants) (Morris, 1976) prevent nuclei from moving through the cytoplasm but do not prevent nuclear division. Partial inactivation of *nudF* has been shown to affect markedly the symmetry of conidiophore development and the normal progression of cell type formation (Xiang *et al.*, 1995). Also, mutation of the *apsA* gene affects both nuclear migration and development of conidiophores, again indicating a close tie between nuclear positioning and correct conidiophore development (Clutterbuck, 1994; Fischer and Timberlake, 1995). Therefore, perhaps the inappropriate formation of multiple septa in the vesicles of the *nimX<sup>cdc2AF</sup>* mutant impairs normal localization of nuclei, which might then prevent normal development.

Another possibility is that the lack of inhibitory phosphorylation sites on NIMX<sup>cdc2</sup> impairs the coordination between cell cycle regulation and the transition from vesicle extension to the budding mode of growth observed during the formation of metulae. In *Saccharomyces cerevisiae* a checkpoint system exists that monitors bud emergence. This morphogenesis checkpoint feeds information regarding bud emergence to tyrosine phosphorylation
of Cdc28 (nimX\textsuperscript{vcd} homolog) through the activity of the Swel (anK\textsuperscript{wei} homolog) protein kinase (Lew and Reed, 1995; Sia \textit{et al}., 1995) to coordinate bud emergence with the cell cycle. This system is linked to the organization of the cytoskeleton and septin function through a protein kinase cascade that eventually inhibits Cdc28 by tyrosine phosphorylation (Carroll \textit{et al}., 1998; Longtime \textit{et al}., 1998; Barral \textit{et al}., 1999). If such a system were operative in \textit{A.nidulans}, it may become critical during metulae formation as growth of the vesicle stops and multiple buds begin to form on its surface. The \textit{nimX\textsuperscript{vcd}2AF} mutation may prevent normal synchronization between bud formation and cell cycle progression eventually contributing to the developmental defects we have observed.

Previous studies in other multicellular organisms, such as plants and \textit{Drosophila}, have indicated that relatively normal development can proceed when cell cycle regulation has been experimentally modified (Edgar and O’Farrell, 1989, 1990; Hemerly \textit{et al}., 1995; Doerner \textit{et al}., 1996). This is apparently not the case for \textit{A.nidulans} as the current work, and previous studies (O’Connell \textit{et al}., 1992), have demonstrated that either impairment or activation of the cell cycle by manipulation of \textit{nimX\textsuperscript{vcd}} markedly modifies development of conidiophores. Additionally, we demonstrate here that the developmental regulator \textit{brlA} influences not only development but also cell cycle genes. Unlike other model multicellular organisms previously studied, there is therefore tight coupling of cell cycle regulation and some aspects of development in \textit{A.nidulans}. Perhaps this is due to the fact that \textit{A.nidulans}, and other filamentous fungi, change both their mode of growth and the number of nuclei per cell in a developmentally regulated manner (Mirabito and Osmani, 1994). This may necessitate close integration of the developmental program with cell cycle regulation in \textit{A.nidulans}, which is not as important in organisms that have a single mode of growth and contain predominantly uninucleate cells.

Materials and methods

\textbf{Growth of Aspergillus and general techniques}

Growth and processing of \textit{A.nidulans} for mRNA isolation, DNA isolation, protein purification, kinase assays, Western blotting and Northern analysis was as described previously (Osmari \textit{et al}., 1987; Ye \textit{et al}., 1995; Osmani and Ye, 1996). Strains employed include R153 (pyroA4::wA3), A583 (bA1::bA42), TTA292 (bA1::argB2:methG1::alcA::brlA) (Adams \textit{et al}., 1988), FRY-20-1 (nimX\textsuperscript{vcd}2AF::pyroA4:pyrG98::wA3) (Ye \textit{et al}., 1996), U1-108 (Y2A:pabaA1:argB::alcA(pl)::abaA::trpC::staA(pl)::lacZ), U1-121 (bA1::argB2::alcA(pl)::brlA::abaA1::trpC::staA(pl)::lacZ) (kind gift of Bruce Miller). To synchronously induce differentiation of conidiophores a thin mycelial mat was filtered from liquid culture and placed upon an agar plate (Boylan \textit{et al}., 1987) that was incubated at 42°C.

\textbf{Poly(A)\textsuperscript{+} mRNA isolation, cDNA synthesis and isolation of \textit{brlA}-induced \textit{nimE}}

Poly(A\textsuperscript{+}) mRNA was purified using the PolyATtract mRNA isolation system (Promega, Madison, WI). In brief, total RNA was heated for 10 min at 65°C, mixed with biotinylated oligo(dt) probe in 0.5× SSC, and annealed at room temperature. The annealing mixture was then added to one tube of streptavidin–paramagnetic particles that had been washed with 0.5× SSC. After 10 min of incubation at room temperature, the streptavidin–paramagnetic particles were washed four times with 0.1× SSC, and the bound mRNA was eluted with RNase-free water.

Poly(A\textsuperscript{+}) mRNA was isolated from alcA::brlA non-induced and induced cells and cDNA was synthesized from these according to the Clontech PCR-Select\textsuperscript{TM} cDNA Subtraction kit user manual (Clontech, Palo Alto, CA). \textit{brlA}-inducible genes were enriched using the Clontech PCR-Select\textsuperscript{TM} cDNA Subtraction protocol as described (Clontech), cloned into PCR2.1 plasmid and transformed into one shot TOP 10E\textsuperscript{+} cells (Invitrogen, Carlsbad, CA). To isolate \textit{brlA}-induced \textit{nimE} from these clones colony array hybridization screening was used employing full-length \textit{nimE} cDNA as a probe. Positive colonies were then grown and their DNAs isolated for sequence analysis. The 5’ and 3’ ends of \textit{nimE} were also obtained using RACE–PCR according to the Marathon\textsuperscript{TM} cDNA amplification kit protocol (Clontech).

\textbf{DNA sequencing, primer extension and DNA analysis}

DNA sequencing reactions were run in a DNA Thermal Cycle model 9600 (Perkin Elmer, Foster City, CA) using ABI Prism Dye Terminator mixture, and were analyzed using an ABI Prism 377 sequencer (Perkin Elmer). An oligonucleotide complementary to the sequence between 625 and 655 bp of \textit{nimE} was labeled with \textit{[\textsuperscript{32P]}dATP} and used in primer extension and end-labeled primer sequencing assays. The end-labeled primer sequencing reaction was completed in a DNA Thermal Cycle model 480 (Perkin Elmer) using AmpliTaq DNA polymerase (Perkin Elmer). To determine the probability of finding BREs at random in the 2045 bp of \textit{nimX\textsuperscript{vcd}} genomic DNA, a simulation was performed using S-PLUS 4.5 software (Seattle, WA). The frequencies of bases were used to randomize the sequence 1000 times. For each randomization, the number of unique CAAGGG sequences and the number of unique C/A G/A AGGG G/A sequences were identified. This yielded a distribution of randomly generated BRE counts to be compared with the number of BREs found in the 2045 bp of \textit{nimX\textsuperscript{vcd}} genomic DNA. In this simulation, seven or more BREs were found in 17 of the 1000 randomly generated sequences.

\textbf{Electron microscopy}

One thousand spores/ml were inoculated into 3 ml of YAG (or YAGUU for FRY-20) 10 agar and then overlaid onto YAG (or YAGU) for FRY-20-1 plates. Plates were incubated at 42°C until conidiophores were visible under a dissecting microscope (23 h for R153, 43 h for FRY-20 and Fry-20-1). Blocks of mycelia were excised from the top agar, placed immediately into ice-cold Karnovsky’s fixative [0.1 M Sorenson’s phosphate buffer pH 7.0 (SPB), 2% formalin, 4% glutaraldehyde] and incubated overnight at 4°C. Samples were washed twice in 0.1 M SPB, and post-fixed for 30 min in 4% osmium tetroxide at room temperature with rocking. Samples were washed twice in 0.1 M SPB and then dehydrated in a graded acetone series, critical point dried in a Samdri-PVT-3B critical point dryer, mounted onto aluminum stubs and sputter coated for 6 min in a Hummer VI sputter coater. Samples were examined at a 35° angle in a JEOL JSM T-300 scanning electron microscope.

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\textbf{References}


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