Kapl, a non-essential member of the Pse1p/Imp5 karyopherin family, controls colonial and asexual development in Aspergillus nidulans

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Asexual development in the filamentous fungus Aspergillus nidulans is governed by the timely expression and cellular localization of multiple transcription factors. Hence, factors mediating import and export across the nuclear pore complexes (karyopherins) are expected to play a key role in coordinating the developmental programme. Here we characterize KapI, a putative homologue of the Saccharomyces cerevisiae Kap121/Pse1p karyopherin. KapI is a non-essential importin-β-like protein located in the nucleus during vegetative growth and conidiophore development. The ΔkapI phenotype is aconidial with many aerial hyphae. This phenotype can be suppressed under abiotic stress. In this regard, it resembles that of the null allele of the bZIP transcription factor FlbB. However a ΔflbB; ΔkapI double mutant exhibited an additive phenotype with totally impaired conidiation, unresponsive to abiotic stress. In contrast to ΔflbB, the null kapI mutant is not a fluffy-low-bristle expression mutant. Taken together the findings indicate that KapI is required during asexual development, mediating the nuclear transport of factors acting in a different pathway(s) from those involving the upstream developmental activators.

INTRODUCTION

In Aspergillus nidulans, the induction of the key transcription factor (TF), BrlA, activates the central developmental pathway of conidiation (Prade & Timberlake, 1993), which includes the sequential formation of the different cellular modules that form the conidiophore: foot-cell, stalk, vesicle, metulae and phialides (primary and secondary stigmata), and finally asexual spores or conidia. Upstream developmental activators (UDAs) are expressed in vegetative cells, and are involved in sensing environmental stimuli and generating signals that activate brlA expression (Adams et al., 1998; Kües & Fischer, 2006). Mutations in most UDA genes generate a phenotype commonly known as ‘fluffy’, which shows delayed or impaired conidiation (Etxebeste et al., 2009, and references therein). Many UDAs are putative TFs (Adams et al., 1998; Etxebeste et al., 2008; Seo et al., 2006; Wieser & Adams, 1995). The best-known member of this family is the bZIP-type TF FlbB, which is localized in the most apical nucleus of vegetative hyphae, and remarkably, in the Spitzenkörper (Etxebeste et al., 2008, 2009; Garzia et al., 2009). However, the mechanisms which regulate FlbB function, and that of other UDAs, remain to be clarified. In the case of those which are TFs, this includes nuclear transport.

In eukaryotes, macromolecule trafficking between cytoplasm and nucleus involves nuclear import and export pathways. This bidirectional transport occurs through nuclear pore complexes (NPC; Fried & Kutay, 2003), which are embedded in the nuclear envelope. Karyopherins are a family of nuclear transporters involved in this selective and active transport (Stewart, 2007). Most karyopherins belong to the superfamily defined by importin-β, and 22 and 15 such proteins have been identified in mammals and Saccharomyces cerevisiae, respectively (Mosammaparast & Pemberton, 2004; Strom & Weis, 2001). The genome of Aspergillus nidulans is predicted to contain 14 importin-β-type coding genes (Espeso & Osmani, 2008).

Our previous work showed noteworthy similarities between the FlbB and Pap1 or Yap1, two bZIP-type TFs
from Schizosaccharomyces pombe and Saccharomyces cerevisiae (Etxebeste et al., 2009; Glover & Harrison, 1995; Isoyaama et al., 2001). Moreover, FlbB was capable of binding the same target sequences as those recognized by Pap1 at the p25 gene promoter from Schiz. pombe (Etxebeste et al., 2009; Toda et al., 1992). In addition, the FlbB amino acid sequence contained a conserved KRGG motif, also contained within the NLS recognized by karyopherin Kap121p/Pse1p at the S. cerevisiae regulator Pho4p (Kaffman et al., 1998; Timney et al., 2006). This work analyses the hypothetical requirement of the Kap121p/Pse1p homologue from A. nidulans, Kapl, for the nuclear import of FlbB, and describes the role of this karyopherin in polarized hyphal growth, branching and conidiophore development.

METHODS

Strains, standard techniques and culture conditions for A. nidulans. The strains of A. nidulans employed in this study (Table 1) were obtained by crossing or transformation following standard protocols (Etxebeste et al., 2008). Strain BD212 was obtained by meiotic recombination between strains TN02A25 and MAD2159. BD212 was then crossed with BD143, BD167 and BD185, to obtain strains BD223, BD230 and BD231, respectively. Strain MAD2446 expresses protein H1::mCh (hhoA locus; Ramon et al., 2000). Strains MAD2334 and MAD2654 were obtained by the transformation of BD167 (Etxebeste et al., 2008) and MAD2446, respectively, with the fusion PCR products to generate C-terminal mCherry or gfp-tagging of the Kapl protein by gene replacement (Etxebeste et al., 2008; Yang et al., 2004).

Phenotypic analyses under stress growth conditions were done using Aspergillus minimal medium (MMA) as in Etxebeste et al. (2009). Phenotypic analyses under stress growth conditions were done using Aspergillus minimal medium (MMA) as in Etxebeste et al. (2009). Moreover, FlbB was capable of binding the same target sequences as those recognized by Pap1 at the p25 gene promoter from Schiz. pombe (Etxebeste et al., 2009; Toda et al., 1992). In addition, the FlbB amino acid sequence contained a conserved KRGG motif, also contained within the NLS recognized by karyopherin Kap121p/Pse1p at the S. cerevisiae regulator Pho4p (Kaffman et al., 1998; Timney et al., 2006). This work analyses the hypothetical requirement of the Kap121p/Pse1p homologue from A. nidulans, Kapl, for the nuclear import of FlbB, and describes the role of this karyopherin in polarized hyphal growth, branching and conidiophore development.

Table 1. Strains used in this study

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<tr>
<th>Strain</th>
<th>Genotype</th>
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<td>TN02A3</td>
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<td>MAD2654</td>
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<td>BD231</td>
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RESULTS

Kap1 is related to importin 5 and Kap121p/Pse1p nuclear transporters

In silico searches had already identified gene kap1/AN5717.3 as the putative homologue of the \textit{S. cerevisiae} nuclear transporter Kap121p/Pse1p (Espeso & Osmani, 2008). The AN5717.3 locus maps within contig 98 of chromosome V, encoding a 1096 amino acid protein called Kap1. An importin-\(b\) N-terminal domain (Pfam, PF03810) is predicted between amino acids 30 and 111 (grey rectangle in Fig. 1a), which might be involved in RanGTPase interaction (Chook et al., 2008).

\textbf{Isolation and manipulation of nucleic acids.} DNA and RNA samples for Southern and Northern blot experiments were isolated and manipulated as described previously (Etxebeste et al., 2008; Garzia et al., 2009).

& Blobel, 1999). Domain searches also predicted the presence of five HEAT (huntingtin-elongation-A subunit-TOR) motifs (Groves et al., 1999) distributed throughout Kapl as indicated in Fig. 1a (dark rectangles). This agrees with reports describing members of the importin-β superfamily to contain multiple HEAT repeats (Petosa et al., 2004).

Phylogenetic analyses revealed that Kapl homologues are found among eukaryotes ranging from fungi to metazoans. Three main branches are clearly differentiated from the phylogenetic tree (Fig. 1b). One corresponds to importin 5 (Imp5) karyopherins from higher eukaryotes. The remaining two branches arise from a common origin and differentiate yeast Kap121p/Pse1p-like proteins and filamentous fungal Kapl-like proteins. The high degree of amino acid sequence conservation among these proteins (Supplementary Fig. S1), with a similarity higher than 50% in all cases, suggests that the mechanisms governing Pse1p/Imp5/Kapl nuclear import pathways are highly conserved. However, while Pse1p has been described as an essential nuclear transporter of S. cerevisiae (Seedorf & Silver, 1997), the strain carrying the null allele of kapl in A. nidulans was viable (Fig. 2a).

Kapl has a role in polar growth and branching

The Δkapl strain showed a significantly lower extension rate ($K_r$ 215.9 ± 3.3 μm h⁻¹) than the wild-type strain ($K_r$ 261.8 ± 2.9 μm h⁻¹), representing a 17.5% reduction (Table 2, Fig. 2a). An analysis of the branching frequency, measured as the average length of hyphae between any two branches ($G_m$ μm), showed that the mutant strain had about twice the branching frequency of the wild-type, both in germlings ($G_m$; Table 2, Fig. 2b) and at the periphery of mature colonies ($G_m$; Table 2, Fig. 2c). $G_m$ decreased from 82.9 ± 1.6 μm in the wild-type to 38.7 ± 0.6 μm in the mutant strain ($n$=20 germlings for each strain), while $G_m$ decreased from 303.9 ± 15.6 μm to 150.8 ± 6.2 μm ($n$=10 hyphae in two colonies of each strain). In order to determine whether the increase observed in the branching frequency could account for the recorded differences in $K_r$, alone without effecting changes in specific growth rate ($\mu$), this kinetic parameter was measured in both wild-type and Δkapl strains. The mutation resulted in a 20% reduction of $\mu$, from 0.0852 ± 0.0169 h⁻¹ in the wild-type to 0.0687 ± 0.0079 h⁻¹ in the Δkapl strain (Table 2). Therefore, the reduced radial extension rates of Δkapl mutant colonies was due to the combined effect of increased branching rate and reduced specific growth rate.

Kapl is required for proper asexual development

Although some conidiophore development was detectable at the centre (aged part) of Δkapl colonies after 72 h of
culture, their pattern of conidiation was notably reduced (Fig. 2a, Fig. 3, first row). Conidia production was reduced by two orders of magnitude compared to the isogenic wild-type strain \((2.18 \times 10^5 \pm 0.76 \times 10^5 \text{ and } 3.97 \times 10^5 \pm 0.56 \times 10^5\) conidia cm\(^{-2}\) respectively; \(n=5\) for each strain).

Since the conidiation pattern of \(\Delta kapI\) was similar to that shown by mutants from the upstream developmental pathway of conidiation (Etxebeste et al., 2008, 2009; Garzia et al., 2009), we analysed the aconidial phenotypes of single and double null \(kapI\) and \(flbB\) strains on solid media under different stress conditions which are known to promote conidiation (Etxebeste et al., 2008, 2009; Garzia et al., 2009). In synthetic complete minimal medium, both \(\Delta flbB\) and \(\Delta kapI\) strains showed an aconidial phenotype, although the former was more prominently fluffy (Fig. 3, first row). A similar conidiation pattern of both \(\Delta flbB\) and \(\Delta kapI\) single mutant strains was observed when the concentration of carbon or nitrogen was reduced to 1/10 (Fig. 3, rows 2–4). Divergent responses were observed under salt stress: KCl reversion of the \(\Delta flbB\) aconidial phenotype (Etxebeste et al., 2008) was not observed in the \(\Delta kapI\) colony, but conidiation was induced in both strains in the presence of an extracellular excess of dihydrogen phosphate anion (Fig. 3, rows 3 and 6). Under osmotic stress, induced by addition of sorbitol or sucrose, the \(\Delta kapI\) strain did not show the autolytic phenotype exhibited by the \(\Delta flbB\) mutant in the central region of the colony. Conversely, conidiation was induced (Fig. 3, rows 7 and 8). Under the conditions analysed, the double (\(\Delta kapI\); \(\Delta flbB\)) strain showed an additive phenotype, displaying an extreme aconidial phenotype even in high-phosphate medium. Under osmotic stress, the central autolytic region was also visible in the double null as in the single \(\Delta flbB\) strain.

The conidiation patterns of \(\Delta kapI\) and double mutant strains were also assessed in liquid cultures (see Methods) (Fig. 4). Cultures transferred to MMA maintained an undifferentiated vegetative growth pattern (Fig. 4, first row). Under glucose starvation, both \(\Delta kapI\) and wild-type strains generated simplified conidiophores (Etxebeste et al., 2008; Garzia et al., 2009; Fig. 4, second row), consisting of phialide-like structures with a single conidium at their end; however, the \(\Delta flbB\) mutant produced narrow hyphae, approximately 1.5 \(\mu m\) in diameter, from which single conidium-like structures rarely emerged (Fig. 4, second row; Etxebeste et al., 2008).

Nitrogen starvation revealed additional phenotypic differences between \(\Delta flbB\) and \(\Delta kapI\) strains. Whereas no conidiation response and an autolytic phenotype were found in \(flbB\) mutants (Fig. 4, third row; Etxebeste et al., 2008), the \(\Delta kapI\) strain produced anomalous conidiophores in which some metulae were abnormally lengthened and did not differentiate into phialides (Fig. 4, third row). Salt stress (0.6 M KCl) induced the formation of fully developed conidiophores in the wild-type strain while the \(\Delta kapI\) strain could not induce conidiation, as occurred with all \(flbB\) mutants previously tested (Fig. 4, fourth row; Etxebeste et al., 2008). On the other hand, for the wild-type and mutant strains, addition of 0.5 M dihydrogen phosphate anion caused the formation of conidiophores with abnormally swollen vesicles, twice the size of those found in standard medium (Fig. 4, fifth row; \(n=20\)). Most notably, under any growth condition, cells from the double mutant strain did not differentiate into conidiophores, implying the requirement of both FlbB and Kapl for the development of any class of conidiating structures. The additive phenotype of the double null strain in either solid or liquid media suggested that FlbB and Kapl might be acting on different regulatory pathways mediating conidiophore development.

### FlbB nuclear import does not require Kapl

In agreement with its predicted karyopherin function, Kapl showed a nuclear localization (Fig. 5a). The Kapl::GFP fusion protein had a distribution rather similar to that described for the *S. cerevisiae* Pse1p karyopherin, as punctate accumulations in the nucleus, some of which were probably in close relationship with the nuclear envelope (Seedorf & Silver, 1997). All nuclei displayed the same localization for Kapl::GFP, predominantly nuclear, with some fluorescence also appearing in the cytoplasm (Fig. 5a).

Given that preliminary results pointed to FlbB as a possible cargo for Kapl (see Introduction), we analysed the localization of the chimeric FlbB::GFP protein in a \(\Delta kapI\) background. Similar localization of FlbB::GFP was observed in either wild-type or \(\Delta kapI\) backgrounds (Fig. 5b). Kapl (as a Kapl::mCh fusion protein) localization barely coincided with that of FlbB::GFP (Fig. 5c). Kapl located mainly at the nuclear periphery of the most apical nucleus (see graph in Fig. 5d), where nuclear FlbB is predominantly observed. In addition, this karyopherin was absent from the tip of the cell, where FlbB also accumulates (Fig. 5e). Neither pull-down assays nor bimolecular fluorescence complementation (BiFC) analyses provided any evidence of interaction between Kapl and FlbB (not shown). These experiments showed that FlbB nuclear import and tip accumulation were independent of the Kapl transport pathway.

### Table 2. Radial extension rate \((K)\), hyphal growth unit in germlings \((G_g)\) and at the periphery of the colony \((G_p)\), and specific growth rate \((\mu)\) of wild-type and \(\Delta kapI\) strains

All parameters were calculated as described in Methods and are means ± SEM of multiple measurements as described in Results.

<table>
<thead>
<tr>
<th>Strain</th>
<th>(K) ((\mu m \text{ h}^{-1}))</th>
<th>(G_g) ((\mu m))</th>
<th>(G_p) ((\mu m))</th>
<th>(\mu) ((\text{h}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN02A3</td>
<td>261.8 ± 2.9</td>
<td>82.9 ± 1.6</td>
<td>303.9 ± 15.6</td>
<td>0.0852 ± 0.0169</td>
</tr>
<tr>
<td>(\Delta kapI)</td>
<td>215.9 ± 3.3</td>
<td>33.7 ± 0.6</td>
<td>150.8 ± 6.2</td>
<td>0.0687 ± 0.0079</td>
</tr>
</tbody>
</table>
Role of importin Kapl in hyphal growth and conidiation

Fig. 3. Analysis of conidiation capacity and colony morphology of Δkapl, ΔflbB and double null mutants in solid media. The photographs on the left show the phenotypes of wild-type, Δkapl, ΔflbB and the double null (ΔflbB; Δkapl) strains grown for 72 h at 37 °C on solid MMA (row 1), MMA with 1/10 the normal concentration of glucose (row 2), ammonium (row 3) or nitrate (row 4), and on MMA with KCl (0.6 M; row 5), NaH$_2$PO$_4$ (0.5 M; row 6), sorbitol (1.2 M; row 7) or sucrose (1.0 M; row 8). Scale bar, 2 cm. The bar diagrams on the right show spore production of each strain in each medium assayed (means ± SEM of five replicates).
brlA is expressed in the absence of KapI activity

UDA factors are positively required for brlA transcription (see Introduction) and some of the mutants in this regulatory pathway are called flb due to the fluffy phenotype associated with a low brlA expression level (Wieser et al., 1994). In contrast to ΔflbB (Etxebeste et al., 2008), or to other flb mutants such as ΔflbE (Garzia et al., 2009), we detected brlA transcription in the ΔkapI deletion strain (Fig. 6). This result supports our assumption that kapI is not a member of the flb gene class, and therefore strongly backs our conclusion that KapI must exert its role on the regulation of conidiation by acting in a pathway different from that mediated by the Flb proteins.

Cellular localization of KapI is maintained during conidiophore development

As KapI is required for conidiophore development, we followed KapI::GFP localization in the different cell modules that form the conidiophore: stalk, vesicle, metulae, phialides and conidia (Fig. 7). For this purpose we constructed a strain expressing both KapI::GFP and the histone H1 fused to the monomeric Cherry fluorescent protein (mCh). We used H1::mCh as an in vivo positional marker of nuclei. In our conditions for induction of asexual development (Etxebeste et al., 2009), we tracked the distributions of nuclei during the different stages of conidiophore morphogenesis, which...
Fig. 5. Distribution of KapI and FlbB in vegetative cells. (a) KapI:GFP localization in nuclei and cytoplasm from vegetative hyphae. Scale bar, 5 µm. (b) FlbB::GFP localization (left panels) compared to that observed in a ∆kapI genetic background (right panels). The arrowhead and the arrow indicate the tip and the nucleus, respectively. Scale bar, 5 µm. (c) KapI::mCh (red) and FlbB::GFP (green) localization in vegetative hyphae. Vertical bars and numbers in the bottom right panel indicate the start and end points for the line profiles shown in the graphs in (d) and (e). Scale bar, 5 µm. (d, e) KapI::mCh (red) and FlbB::GFP (green) relative fluorescence intensities at the hyphal tip and the apical nucleus, respectively.
Fig. 6. brlA expression in vegetative and asexual cycles. brlA expression at 18 h of vegetative growth and 6, 12, 24 and 48 h after the induction of asexual development for ΔkapI and wild-type isogenic strains. Loading control is shown by the ethidium-bromide-stained ribosomal 40S subunit.

Fig. 7. KapI localization in conidiophores. (a) Localization of KapI::GFP (green) and H1::mCh (red; positional marker for nuclei) in an immature conidiophore bearing a stalk, vesicle and metulae. KapI is accumulated mainly at the nuclear envelope but is not excluded from the nucleoplasm. The region of fluorescence exclusion seen in most nuclei is probably the nucleolus. ‘Vac’ in the top left panel indicates the vacuole-like structures in the stalk and vesicle. Scale bar, 10 µm. (b) Localization of KapI::GFP (green) and H1::mCh (red) in a mature conidiophore. KapI is also observed in phialides and conidia. The arrows marked V, M+P and C in (a) and (b) indicate the vesicle, metulae plus phialides and conidium, respectively, that are shown magnified in (c). Scale bar, 20 µm. (c) Magnification of the differentiated cells marked in (a) and (b). Scale bars: 4 µm for the pictures showing the vesicle, 5 µm for metulae and phialides, and 3 µm for the conidium.

were comparable to those observed before using electron microscopy (Mims et al., 1988). Nuclei were evenly distributed along the stalk and concentrated at the vesicle due to the presence of large vacuoles (Fig. 7a). In these conditions, KapI was observed in all the nuclei, from the multinucleate stalk and vesicle to the mononucleate metulae, phialides and conidia, in all cases following a similar location to that described above in vegetative cells (see Fig. 7b for a general view and Fig. 7c for higher magnifications). The presence of KapI in the nucleus during all the stages of conidiophore formation is consistent with the requirement of this nuclear transport pathway during the process of conidiation.
DISCUSSION

In this work we report the characterization of KapI, the A. nidulans Imp5/Kap121p/Pse1p karyopherin homologue. This family of transporters controls general and varied processes in all eukaryotic organisms. In humans, Imp5 regulates, for example, neuronal differentiation and degeneration by the import of p35 protein (Fu et al., 2006), chromosome structure by the import of core histones (Baake et al., 2001), and specific DNA recombination processes through RAG-2 binding (Ross et al., 2003). In yeast, Kap121p/Pse1p is an essential protein that regulates multiple pathways such as the phosphate stress response via Pho4p import (Kaffman et al., 1998), the oxidative stress response via Yap1 (Isoyama et al., 2001), membrane biogenesis by Pdr1 (Delahodde et al., 2001), and both mating response and pseudohyphal transition mediated by Ste12 (Leslie et al., 2002). From our study in A. nidulans, evidence of different levels of control has emerged. Kap does not exclusively participate in essential import or export pathways in this fungus. However, it may participate in the regulation of the nuclear transport of an undetermined protein required for polarized growth. Our recent work on this topic showed the involvement of the TF FlbB not only in conidiation but also in branching. While FlbB is involved in branching, but not in extension growth (Etxebeste et al., 2009), Kap is required for both processes; nevertheless Kap is not essential in either process.

Notably, KapI also plays an important role in asexual differentiation. Although ΔkapI is not an flb mutant, as brlA is expressed, the absence of this nuclear importer in the fungal cell largely prevents normal conidiophore production. Differences were found between the aconidal phenotype of null ΔkapI and the fluffy phenotype of ΔflbB in response to different conidiation-inducing stimuli. Moreover, the ΔkapI mutant has in common with other fluffy mutants such as ΔfluG that it does not participate in the autolytic process in which FlbB is involved in response to osmotic stress induced by sorbitol or sucrose (Etxebeste et al., 2009). These results lead us to propose that KapI and FlbB may act in different regulatory pathways, or that the latter is transported to the nucleus by more than one karyopherin. The additive phenotype of the double null (ΔkapI; ΔflbB) mutant, in which conidiation is completely blocked, suggests that both pathways must be coordinated to activate conidiation in response to inducing media conditions.

In diverse transport processes, Kap121p/Pse1p requires coordination with Kap123p (Isoyama et al., 2001; Rout et al., 1997; Schaper et al., 2005; Seedorf & Silver, 1997). In A. nidulans, a redundancy between KapI and Kap (Kap123p putative homologue) could be also proposed. However, the absence of evidence precludes the assumption of a concerted action between those two karyopherins in the conidiation regulatory pathway, as the ΔkapI strain does not display an aconidal phenotype and no interaction has been detected between KapI and FlbB in pull-down analyses (our unpublished results).

It is important to underline the high number of TFs that participate in conidiation signalling, either in the UDA pathway or in the central regulatory pathway governed by brlA (Kües & Fischer, 2006). Four putative TFs have been previously characterized in the first pathway: FlbB, FlbC and FlbD (Adams et al., 1998), and SfgA, a hypothetical repressor of Flb factors in the genetic cascade that leads to brlA induction (Seo et al., 2006). The results obtained in this work lead us to propose that KapI is not the importer of FlbB. In addition, as brlA is expressed in the null ΔkapI background but not in flbD null (Wieser & Adams, 1995) or flbC mutants (Wieser et al., 1994), we discard the possibility of an exclusive import of those two TFs by KapI.

The role of KapI is also extended to the production of the different modules that form the conidiophore. We observed its presence at the nuclear envelope in stalks, vesicles, metulae, phialides and conidia. It may be postulated that KapI could regulate general pathways during these stages, but also conidiation-specific processes, especially at the metula stage, where its participation seems to become essential. Under nitrogen-starvation conditions in liquid cultures, the ΔkapI mutant had abnormally elongated metulae without any differentiation into phialides, while the wild-type strain produced fully developed conidiophores. We have recently described that a control point already exists at the metula–phialide transition stage and that conidiophore development in a wild-type strain can be reverted by changing media conditions, generating hypha-like structures that can be indefinitely elongated (Etxebeste et al., 2009). Besides BrlA, other previously characterized TFs regulate the central developmental cascade of conidiation, as for example the bZIP-like AbaA (Sewall et al., 1990) or StuA (Dutton et al., 1997). Their mutants produce aberrant conidiophores altered at the metula or phialide stage. KapI could participate in the control point proposed at the metula stage, having a key role in the import of any of these TFs or unidentified additional TFs. Future identification of KapI interactors will constitute a novel approach to the understanding of this important dispersion mechanism of economically and medically important Aspergillus species.

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REFERENCES


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