The *Aspergillus nidulans* putative kinase, KfsA (kinase for septation), plays a role in septation and is required for efficient asexual spore formation

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Abstract

In *Aspergillus nidulans* nuclear division and cytokinesis are coupled processes during asexual sporulation. Metulae, phialides and conidia contain a single nucleus. Here we describe the role of a putative *Saccharomyces cerevisiae* Kin4-related kinase, KfsA (kinase for septation) in the control of septum formation in *A. nidulans*. The *kfsA* deletion caused an increase in the number of conidiophores with septa in their stalks from 20% in wild type to 60% in the mutant strain. Interestingly, 7% of metulae contained two nuclei and the corresponding phialides remained anucleate, suggesting septum formation before proper segregation of nuclei. This points to a checkpoint control of KfsA, which prevents septum formation before nuclear separation. KfsA localized to the cortex and septa in hyphae and in conidiophores but not to the spindle-pole bodies, as it was shown for Kin4 in yeast. KfsA appeared at septa after actin disappeared, suggesting an additional role of KfsA late during septum formation.
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1. Introduction

Cytokinesis is a fundamental process in all living cells. If cytokinesis is followed by cell–cell separation, cells will double their number with each division. This is the case in e.g. *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Hence, cytokinesis has to be a well-controlled process in yeasts (Moseley and Goode, 2006). It is crucial, that cells do not initiate spindle disassembly and cytokinesis before chromosomes have been properly segregated. Cells have developed several mechanisms to delay exit from mitosis and initiation of the next cell cycle in response to

defects in late mitosis. Best studied are two homologous kinase cascade signaling pathways in budding and fission yeast, named the mitotic exit network (MEN) and septation initiation network (SIN), respectively (Bosl and Li, 2005; Krapp et al., 2004; McCollum and Gould, 2001). A component of the MEN signalling pathway in *S. cerevisiae* is the kinase Kin4 (D'Aquino et al., 2005; Pereira and Schiebel, 2005). The gene was discovered in a genome-wide analysis of genetic interactions, where it genetically interacted with *KAR9*, a gene involved in the dynein spindle-alignment pathway (Miller and Rose, 1998; Miller et al., 1999; Tong et al., 2004). The gene was later also identified in a screen for negative regulators of the MEN network (D'Aquino et al., 2005). Further studies showed that Kin4 is not a component of the spindle alignment pathway itself but indeed is a component of the MEN. Deletion of *KIN4* did not cause any discernible phenotype under normal growth conditions, but allowed mitotic exit with a misaligned anaphase spindle (D'Aquino et al., 2005; Pereira

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and Schiebel, 2005). The protein localized to the cortex of the mother cell and during midanaphase asymmetrically to the spindle pole body located in the mother. In late anaphase it strongly associated with the bud neck. Whereas the role of Kin4 in mitotic exit inhibition in cells with a misaligned spindle has been studied in great detail, it is not clear yet how the signal is transmitted from the spindle pole body to the bud neck and especially which role Kin4 plays at this place.

If cytokinesis is not followed by cell separation, the result will be a tissue or, as in the case of filamentous fungi, a septated hypha. As a difference to animal cells, in filamentous fungi mitosis may occur independent of septation. As a result, fungal compartments of many fungal species are multinucleate. Hence, cytokinesis does not necessarily require a checkpoint control, given that only a single nucleus suffers a problem during late mitosis. However, there is good evidence that such a checkpoint control exists. When *Aspergillus nidulans* hyphae are treated with sublethal concentrations of hydroxy urea to induce DNA damage, wild-type cells delay septation (Harris and Kraus, 1998).

An important question is how defects in mitosis are transmitted to future septation sites along the cortex. That there is a connection between the nuclei and septa is suggested by the fact that spindle pole bodies seem to play an important role in septum positioning. Many proteins found at the spindle pole body seem to affect septation. For instance the spindle pole body protein SnaD affects the timing of mitosis and septation (Liu and Morris, 2000). Interestingly, the *snaD* mutants have a defect in conidiation where the timing of septation is critical for cellular development. Another example is BimG PP1. GFP studies indicate that the spindle pole body protein BimG locates to the site of septation just after mitosis and follows the contractile ring as it divides the cell (Fox et al., 2002). Temperature shift experiments support the idea that BimG plays a direct role in septation, but it is difficult to exclude potential indirect effects caused by perturbance of mitosis.

In many filamentous fungi, such as *A. nidulans,* it has to be considered that the syncytial stage is replaced by a strict coupling of mitosis to cytokinesis during development. *A. nidulans* reproduces with asexual conidiospores, which are generated at conidiophores. These structures consist of a conidiophore stalk with a vesicle, from which metulae emerge. Metulae produce 2–3 phialides, each, which in turn continuously generate conidiospores. Metulae, phialides and spores are single cells, which contain a single nucleus each. Metulae and phialides, and the mechanism of reproduction, resemble the pseudohyphal growth form of *S. cerevisiae* and thus it is likely that a mitotic exit network is important for proper production of spores in *A. nidulans* or other filamentous fungi.

Here, we characterized a putative kinase, KfsA, with similarity to *S. cerevisiae* Kin4 and found that it localizes to the cortex of hyphae, and to septa but not to spindle pole bodies. GFP-KfsA appeared at septa after the con-

tractile actin ring was formed. The kinase is necessary for proper asexual spore formation.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

Supplemented minimal and complete media for *A. nidulans* were prepared as described, and standard strain construction procedures are described by (Hill and Käfer, 2001). A list of *A. nidulans* strains used in this study is given in Table 1. Standard laboratory *Escherichia coli* strains (XL-1 blue, Top 10 F′) were used. Plasmids are listed in Table 2.

2.2. Molecular techniques

Standard DNA transformation procedures were used for *A. nidulans* (Yelton et al., 1984) and *E. coli* (Sambrook and Russel, 1999). For PCR experiments, standard protocols were applied using a capillary Rapid Cycler (Idaho Technology, Idaho Falls, USA) for the reaction cycles. DNA sequencing was done commercially (MWG Biotech, Ebersberg, Germany). Genomic DNA was extracted from *A. nidulans* with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA was isolated with TRIzol® GibcoBRL Life technologies, Paisley, Scotland, UK) according to the manufacturer's protocols. DNA analyses (Southern hybridizations) were performed as described by (Sambrook and Russel, 1999).

2.3. Deletion of kfsA

The *kfsA* flanking regions were amplified by PCR using genomic DNA and the primers Snf1_5 (5′-ACCCAACT ACTAGTAACGATTGTT-3′) and Snf1_5-*Sfi*I_rev (5′-G GCCATCTAGGCCGGATTTATGAAACGTCTTCTC GT-3′) for the upstream region of *kfsA* and Snf1_3-*Sfi*I (5′-GGCCTGAGTGGCCCCAGCTGCTCTATATCTTGC TT-3′) and Snf1_3_rev (5′-CGAAGCTTAATATACC TGCTACCAC-3′) for the downstream region and cloned into pCR2.1-TOPO, to generate pAB6 and pAB7, respectively. In a three-fragment-ligation the *argB*-gene from plasmid pSK70 was ligated between the two *kfsA*-flanking regions, resulting in vector pAB10. The deletion cassette was amplified with the primers Snf1_5 and Snf1_3_rev and the resulting PCR-product transformed into the arginin-auxotrophic *A. nidulans* strain SRF200. Among 30 transformants, analyzed by PCR, four displayed homologous integration of the deletion cassette at the *kfsA* locus. As primers for the indicative PCRs we used oligonucleotides derived from the *argB* gene: arg5 (5′-CTGAGAAAT GATTCGTGAATG-3′) and arg3 (5′-GACTCTCCTCAT TCCATAC-3′), the *kfsA* internal primers Snf1_for (5′-C TGCGAGATCTATCTCATCCG-3′) and Snf1_rev (5′-T CGTTTTCGGGGATCAGG-3′) and primers outside the *kfsA* gene deletion construct Snf1_5for_outside (5′-CTG

Table 1
A. nidulans strains used in this study

Strain	Genotype	Source
SRF200	*pyrG89; ΔargB::trpCΔB; pyroA4; veA1*	(Karos and Fischer, 1999)
TN02A3	*pyrG89; pyroA4, argB2, nkuA::argB*	(Nayak et al., 2005)
SSNI1	SRF200 transformed with pDC1 (*argB*)	N. Schier, Marburg
A. nidulans actin-GFP	*pyrG89; wA3; pyroA4; alcA(p)-α–actin-GFP::pyrG*	S. Osmani (Ohio State University, USA)
SJW02	*wA3; pyroA4; alcA(p)::GFP::tubA, ΔargB::trpCΔB*	(Toews et al., 2004)
SAB3	Deletion of *kfsA* in SRF200; *pyrG89; ΔargB::trpCΔB; pyroA4; veA1; ΔkfsA::argB*	This study
SNT11	TN02A3 transformed with pNT3	This study
SKV49	SRF200 transformed with pAB20; *pyrG89; ΔargB::trpCΔB; pyroA4; veA1; alcA(p)::kfsA-sGFP,argB*	This study
SKV51	SAB3 cotransformed with pAB20 and pRG1	This study
SNT3	*A. nidulans* actin-GFP, cotransformed with pAB21 and pI4	This study
SNT18	SJW02 cotransformed with pAB21 and pI4	This study
SNT20	SAB3 cotransformed with pRS31 and pI4	This study
SRS27	*pyrG89;pyroA4; veA1; gpd(p)::stuA(NLS)::sGFP*	(Suelmann et al., 1997)
SNT28	TN02A3 transformed with pRG1	This study

Table 2
Plasmids used in this study

Plasmids	Construction	Source
pCR2.1-TOPO	Cloning vector	Invitrogen (NV Leek, The Netherlands)
pENTR/D-TOPO	GATEway TOPO cloning vector	Invitrogen (NV Leek, The Netherlands)
pCMB17apx	*alcA(p)::GFP*, for N-terminal fusion of GFP to proteins of interest; contains *N. crassa pyr4*	V. Efimov (Piscataway, USA)
pAB6	1 kb 5′-flanking region of *kfsA* with *Sfi*I-site in pCR2.1	This study
pAB7	1 kb 3′-flanking region of *kfsA* with *Sfi*I-site in pCR2.1	This study
pAB10	*kfsA*-deletion construct: flanking regions from pAB6 and pAB7 ligated with *argB* from pSK70	This study
pAB13	*kfsA*-ORF without stop-codon in pENTR/D-TOPO	This study
pAB20	*alcA(p)::kfsA-sGFP, argB; kfsA* from pAB13 via GATEway in pMT-sGFP	This study
pAB21	*alcA(p)::kfsA-mRFP1, argB; kfsA* from pAB13 via GATEway in pMT-mRFP	This study
pMT-sGFP	GATEway Vector, *alcA(p)::cccD*-box (incl. *attR*-sites)::*sGFP, argB*	(Toews et al., 2004)
pMT-mRFP	GATEway Vector, *alcA(p)::cccD*-box (incl. *attR*-sites)::*mRFP1, argB*	(Toews et al., 2004)
pDC1	*argB* from *A. nidulans*	(Aramayo et al., 1989)
pRG1	*N. crassa pyr-4* selectable marker plasmid	(Waring et al., 1989)
pSK70	*argB* with *Sfi*I-sites	(Vienken and Fischer, 2006)
pI4	*pyroA* from *A. nidulans*	(Osmani et al., 1999)
pNT2	1.7-kb *kfsA* fragment in pCR2.1-TOPO	This study
pNT3	1.7-kb *kfsA* fragment in pCMB17apx	This study
pRS31	GFP expression plasmid containing the gpd promoter, sGFP and NLS of StuA	(Suelmann et al., 1997)

GCA TTC GGTTTGTAGAG-3′) and Snf1_3rev_outside (5′-CCATTACTCAGGACTTAATG-3′) (Fig. 2).

2.4. Tagging of KfsA with GFP and mRFP1

To create an N-terminal fusion construct, a 1.7-kb N-terminal fragment of *kfsA* (starting from ATG) was amplified from genomic DNA with the primers kfsA_Efi_for (5′-GGCGCGCCCGGGATGACTATGATCATTGCTGGC-3′) and kfsA_Efi_rev (5′-TTAATTAATTACGAGACAAGATCGGCATAC-3′) and cloned into pCR2.1-TOPO, yielding pNT2. The *Asc*I-*Pac*I fragment from pNT2 was subcloned into the corresponding sites of pCMB17apx, yielding pNT3. Homologous recombination

of this plasmid into the *kfsA* locus should result in N-terminal GFP fusion of the entire *kfsA* gene under control of the *alcA* promoter. Among 12 transformants of strain TN02A3, 11 displayed the *kfsA*-deletion phenotype (less spores) under repressing conditions (glucose) and wild-type phenotype and similar GFP localization pattern under inducing conditions (glycerol), one of which was named SNT11. PCR analysis confirmed that the construction had integrated at the *kfsA* locus.

To create a C-terminal fusion construct, the complete *kfsA*-ORF was amplified with primers GATE_snf1_5for (5′-CACCATGACTAT GATCATTGCTGGC-3′, underlined are the four bases needed for site-directed TOPO-cloning) and GATE_snf1_3rev (5′-GCGGTCGTCCCC

AACTCTG-3′) with the *proof reading* Phusion-Polymerase (Finnzymes, Oy, Espoo, Finnland) and cloned into the vector pENTR/TOPO (Invitrogen, Karlsruhe) resulting in pAB13. Fusion of KfsA with sGFP/mRFP1 at the C-terminus was done with the GATEway cloning system and vector pMT-sGFP (Toews et al., 2004) resulting in vector pAB20 or pMT-mRFP1 (resulting in pAB21), respectively. The plasmids were transformed into the *A. nidulans* strain SRF200. For actin-colocalisation strain SNT3, containing α-actin-GFP was co-transformed with plasmids pAB21 and pI4 (*pyroA*-gene). For microtubule co-localisation strain SJW02 containing GFP-TubA was co-transformed with pAB21 and pI4 to yield strain SNT18.

2.5. Electron microscopy

For scanning electron microscopy (SEM), colonies grown on MM plates were transferred with a piece of agar into 5% glutaraldehyde for fixation. After several washings with water, the pieces were transferred to glycol-monoethyl ether and incubated overnight at room temperature. They were then transferred to water-free acetone and critical point dried. The samples were then sputter coated with gold and observed with a Hitachi S-530 SEM.

2.6. Light/fluorescence microscopy

For live-cell imaging of germlings and youg hyphae, cells were grown on cover slips in 0.3 ml of medium, either MM + 2% glycerol or MM + 2% ethanol (or threonine). Cells were incubated at 30 °C for 15 h. For pictures of conidiophores Aspergillus strains were inoculated on microscope slides covered with MM + 2% glycerol + 0.8% Agarose and grown at 30 °C for three days. Septa and nuclei were stained with Calcofluor white (Sigma) and Hoechst33342 (Sigma), respectively. Images were captured at room temperature using an Axiophot microscope (Zeiss, Jena, Germany), a Planapochromatic 63× or 100× oil immersion objective lens, and a 50 W Hg lamp. Fluorescence was observed using standard FITC and Rhodamine filter sets. Images were collected and analyzed with a Hamamatsu Orca ER II camera system and the Wasabi software (version 1.2). For Z-stacking we used the Zeiss Apotome system (Axiovision).

3. Results

3.1. KfsA is a putative protein kinase conserved in filamentous fungi

We were interested in the carbon metabolism of *A. nidulans* during asexual and sexual development and analyzed the *A. nidulans* genome for homologues of the *S. cerevisiae* kinase Snf1. We identified seven putative protein kinases, one of which (AN0822.1) displayed best similarity to the *Colletotrichum gloeosporides* Snf1 homologue (Goodwin and Chen, 2002). However, sequence similarity was mainly found in the conserved kinase domain. Deletion experiments and tests for carbon sensing in the deletion mutant revealed that this *A. nidulans* kinase does not play a role in carbon metabolism but in septation. Therefore, we are going to describe in this work the detailed analysis of the gene without further discussing a role in metabolism. We named this new kinase KfsA (= kinase for septation).

The genomic sequence of *kfsA,* as deposited in the genome database under the Accession No. AN0822.1, was confirmed by PCR-amplification of small genomic fragments and subsequent sequencing (results not shown). To determine the intron–exon borders, we amplified corresponding cDNAs by RT-PCR. Comparison of genomic and cDNA sequences revealed the presence of two introns, one in the 5'-region of the gene (67 bp) and another one in the middle of the gene (64 bp). The predicted KfsA protein comprises 1091 amino acids and has a predicted molecular mass of 119 kDa and a calculated isoelectric point of 9.4. A protein kinase domain of about 350 amino acids length was predicted with different pattern prediction tools at (www.expasy.org) (Fig. 1).

When we analyzed if KfsA is evolutionarily conserved, we searched the protein databases for similar proteins. Whereas the filamentous ascomycetes *Aspergillus terreus, Aspergillus fumigatus, Aspergillus oryzae, Gibberella zeae, Phaeosphaeria nodorum, Chaetomium globosum, Magnaporthe griseae,* and *Neurospora crassa* contain proteins with identities between 70 and 43 %, proteins in *S. cerevisiae, S. pombe* or *Candida albicans,* the basidiomycetes *Ustilago maydis*, and *Coprinus cinereus* or the zygomycete *Rhizopus oryzae* shared only similarities in their kinase domains. Only the *KIN4* gene from *S. cerevisiae* has been characterized experimentally (D'Aquino et al., 2005; Pereira and Schiebel, 2005). When the KfsA sequence outside the
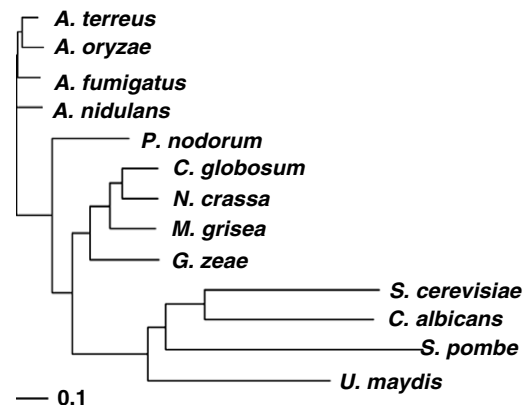

Fig. 1. Relatedness analysis of KfsA and other similar fungal kinases. The Accession Nos. of the proteins are as follows: *C. globosum* (CH408029); *N. crassa* (AABX01000021); *M. grisea* (XM363270); *A. terreus* (CH476594); *A. oryzae* (AP007151); *A. fumigatus* (AAHF01000004); *A. nidulans* (AACD01000013); *P. nodorum* (CH445343); *S. cerevisiae* (NC001147); *C. albicans* (AACQ01000127); *G. zeae* (AACM01000034); *U. maydis* (AACP01000185); *S. pombe* (Q9HFF4). The analysis was done with the ClustalW program (http://www.ddbj.nig.ac.jp/search/clustalw-j.html) and the TreeView X software.

kinase domain was used in the BLAST search, only the four Aspergilli produced high-score hits. In one group of fungi (*A. nidulans, A. terreus, A. fumigatus, P. nodorum*) the proteins are about 150 amino acids longer than the ones of a second group (*A. oryzae, G. zeae, C. globosum, M. grisea, N. crassa*). In comparison to KfsA from *A. nidulans*, *S. cerevisiae* Kin4 consists of only 800 amino acids and the similarity is mainly restricted to the kinase domain. Likewise, *S. cerevisiae* Snf1 and Kin4 only display similarity in the kinase domain (43% identity).

3.2. Deletion of kfsA

In order to determine the function of *kfsA* in *A. nidulans*, we deleted the open reading frame from the genome (see Section 2) and confirmed the deletion event by PCR and Southern blotting (Fig. 2). From 30 transformants four showed the right banding pattern and had no additional ectopic integrations. Deletion of the gene was apparently not lethal. All deletion strains appeared with a similar phenotype and did not have any obvious defects in hyphal growth but produced less conidiospores.

The deletion phenotype was confirmed in two other experiments. In one approach we replaced the genomic copy of *kfsA* by a GFP tagged version under the control of the inducible *alcA* promoter, where we tagged the KfsA protein with GFP at the N-terminus (strain SNT11; see
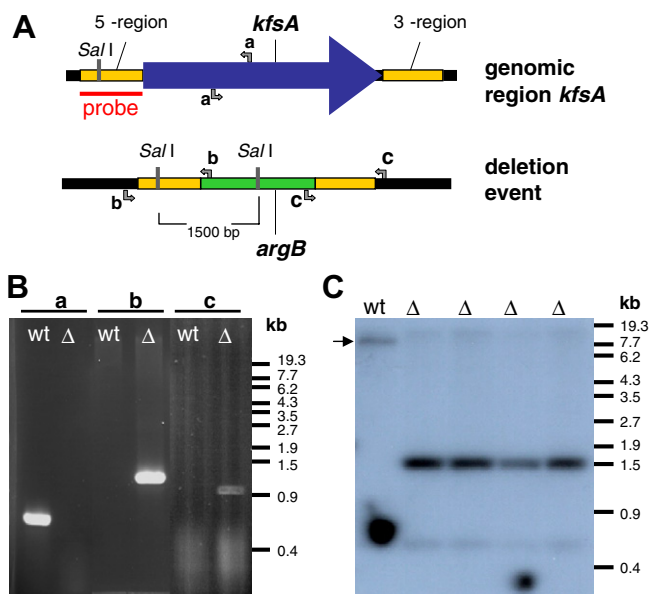

Fig. 2. Deletion of the *kfsA* gene. (A) Scheme of the genomic region in wt and in the deletion strain. (B) Demonstration of the *kfsA*-deletion event via PCR analysis using three oligonucleotide pairs. (a) Snf1_for and Snf1_rev, (b) Snf1_5for_outside and arg5, (c) Snf1_3rev_outside and arg3. Positions of the oligonucleotides are indicated in (A). PCR fragments were separated on a 1% agarose gel and stained with ethidium bromide. (C) Southern blot analysis of a wild type (wt; SRF200) and four *kfsA*-deletion strains. Genomic DNA was isolated, restricted with *Sal*I, separated on a 1% agarose gel, blotted and hybridised with the ^{32}P-labelled probe indicated in (A). The arrow indicates the *kfsA* wild-type band.
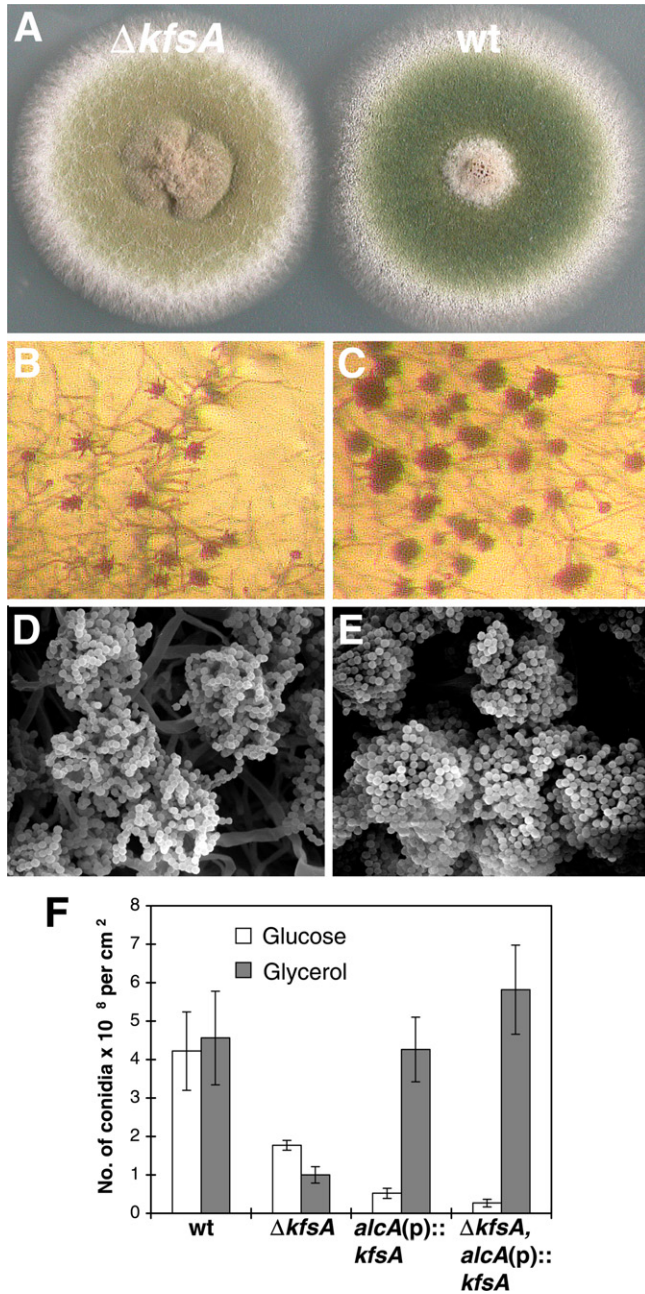
Section 2). In another approach, we introduced full-length *kfsA* in the *kfsA*-deletion strain, where the KfsA protein was tagged with GFP at the C-terminus and produced under the control of the *alcA* promoter (strain SKV51; see Section 2). When grown on glucose the promoter is repressed, when grown on glycerol it is de-repressed and when grown on threonine or ethanol it is de-repressed and activated (Felenbok et al., 2001). Thus different carbon sources allow different expression levels of the protein. When strain SNT11 and SKV51 were grown on glucose, the colonies displayed the similar phenotype as the deletion strain and when grown on inducing media, the wild-type phenotype was restored. The reduced conidiospore production was quantified in several strains (Fig. 3F). These results showed that the observed phenotypic alterations were due to the modification of the *kfsA* locus and not due to other mutations in the genome. When we compared the conidiophores of the *kfsA*-deletion strain or the *kfsA*-down-regulated strain with wild-type conidiophores, we noticed several differences. In wild type the chains of conidiospores form a compact head, whereas the mutants appeared more de-arranged. Furthermore, we observed in more than 60% of the *kfsA*-deletion strain conidiophores septa in the conidiophore stalk whereas in wild-type conidiophores only about 20% had septa (Fig. 4A and B). The phenotype was restored in the complemented mutant strain SKV51. This result suggested a role of KfsA in septation. Therefore we asked whether sepation was also altered in vegetative hyphae. We measured the length of the hyphal compartment in the *kfsA* deletion or wild-type strain but could not find a significant difference (*n* = 200)(results not shown). The number of nuclei per compartment was also not changed.

In *S. cerevisiae*, Kin4 functions in a checkpoint control for spindle miss alignment during cytokinesis (Pereira and Schiebel, 2005). Cytokinesis does not happen before spindles align properly in the budding neck and each daughter cell has received one nucleus. In Δ*kin4* strains, where the checkpoint control is affected, mother cells with two nuclei and daughter cells without a nucleus occur. To investigate whether KfsA serves a similar function in *A. nidulans*, we analyzed nuclear distribution in the *kfsA*-deletion strain by using SNT20, in which nuclei are stained with GFP (Δ*kfsA, gpd(p)::GFP-stuA (NLS)*). This strain showed normal nuclear distribution in hyphae as compared to a control strain (SRS27). However, when we compared nuclear distribution in conidiophores, it looked normal in most conidiophores, but we found a small number of metulae with two nuclei. The corresponding phialides had no nucleus (7%, *n* = 100)(Fig. 5). We could not find such conidiophores in the control strain (SRS27, *n* = 100). Because it is sometimes difficult to obtain high-quality pictures of nuclear distribution in the conidiophore, we confirmed the nuclear distribution defect by microscopic Z-stack analysis. We also found a small number of conidiospores in the *kfsA* deletion strain without any nucleus (12

Fig. 3. Phenotypic effect of *kfsA* deletion. (A) Wild type (SSNI1) and a Δ*kfsA* strain (SAB3) were point-inoculated and incubated for three days at 37 °C. The brighter colour of the Δ*kfsA* strain in comparison to the wild type is due to the reduced number of spores. (B,C) Light microscopic magnification shows an abnormal shape of the conidiophores in the deletion strain. (D,E) Scanning electron microscopic pictures of the conidiophores show, that the spore chains in the deletion strain are not as dense and less ordered as in the wild type. (F) Quantification of the number of conidiospores. Approximately 10^8 spores of each strain, wild type (SSNI1), Δ*kfsA* (SAB3) *alcA(p)::GFP::kfsA* (SNT11) and Δ*kfsA, alcA(p)::kfsA::GFP* (SKV51) were collected in 1 ml water, spread on glucose or glycerol plates, and incubated for 4 days. The conidiospores in 0.5 cm²-fragments of the lawn were counted. The error bars represent the standard deviation of six independent countings.

conidiospores, $n = 500$). In the control strain we found only two conidiospores without a nucleus ($n = 500$). These results indicated a checkpoint control function of
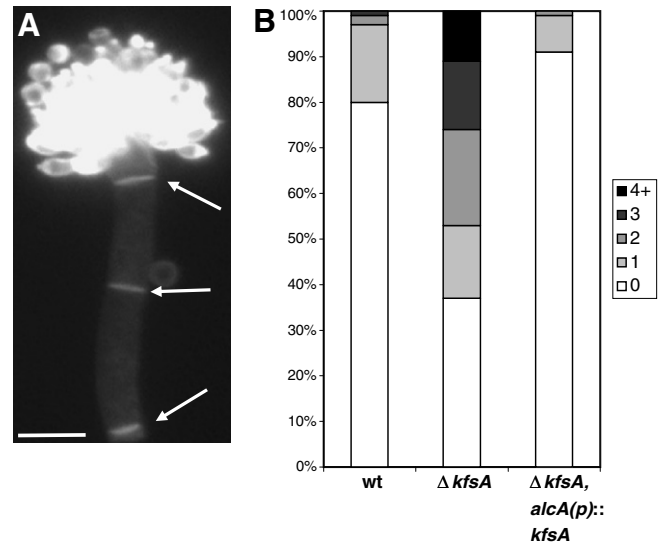

Fig. 4. Septated conidiophores in *kfsA*-mutant strains. (A) Conidiophore stalks in a Δ*kfsA* strain stained with calcofluor white. Septa are indicated with the arrows. Scale bar 10 μm. (B) Quantification of the number of septa in conidiophores ($n = 100$). Wild type (SSNI1), Δ*kfsA* (SAB3) and Δ*kfsA, alcA(p)::kfsA::GFP* (SKV51) were grown on microscope slides covered with MM + 2% glycerol and 0.8% agarose for three days.

KfsA in septation in a similar way than Kin4 in *S. cerevisiae*. To investigate further the role of KfsA in septation, we studied the localization of the protein.

3.3. Localisation of KfsA

To investigate the subcellular distribution of KfsA, we used strain SNT11, in which KfsA was fused at the N-terminus with GFP. The fact that induction of the GFP construct led to wild-type phenotype (above) showed that the fusion protein was fully functional. We expressed the gene with glycerol as carbon source, which leads to low expression levels. The protein specifically localized to all septa and to the cortex, predominately at the cortex behind new septa (Fig. 6A and B). Identical results were obtained when KfsA was tagged at the C-terminus with GFP (SKV49, results not shown). Induction of the promoter with ethanol or threonine and thus overexpression of *kfsA* did not cause any detectable phenotype.

Because the localization pattern resembled somehow the one of actin, we attempted to co-localize KfsA with actin in a spatial and temporal manner. We constructed a strain in which actin was visualized by GFP and KfsA by mRFP1 fluorescence (strain SNT3). At the hyphal cortex, the two proteins did not co-localize. KfsA moved along the cortex in both directions and was clearly different from the dynamics of the actin patches (see movies)(Fig. 6B). In order to analyze the role of KfsA further, we asked whether KfsA accumulation at septa occurred before the visual accumulation of actin or after. In a time-course experiment, where we followed septation, actin and KfsA localization, we found that the place of a new septum was first labelled by actin (Fig. 6C). After 5 min, actin began to
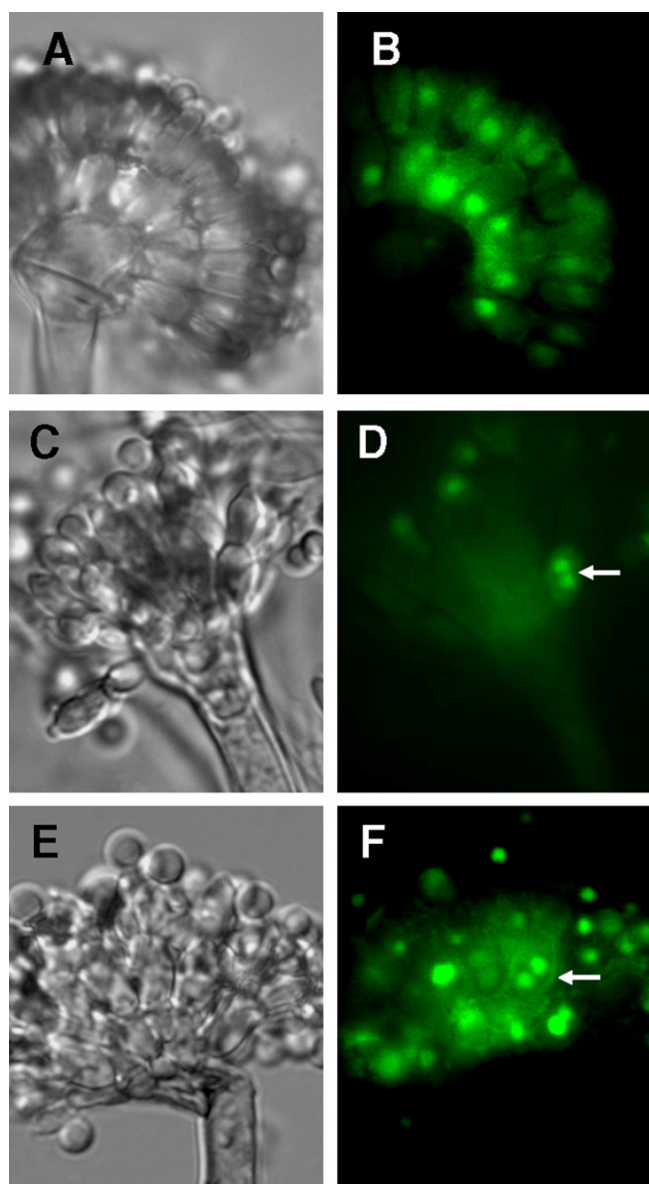
Fig. 5. Nuclear distribution in conidiophores of wild type and a Δ*kfsA* strain. DIC (A,C,E) and nuclear-GFP signal (B,D,F) in conidiophores of strain SRS27 (*gpd(p)::stuA(NLS::GFP)*) (A,B) and strain SNT20 (Δ*kfsA, gpd(p)::stuA(NLS)::GFP*) (C–F) grown on MM glucose plates. Arrows indicate two nuclear-GFP signals in metulae. Scale bar 10 μm.

diffuse from the septa and a weak signal band of KfsA-mRFP1 was observed at the same place. After 7 min actin labelling disappeared completely, while KfsA remained at the septa. Our observations suggest that KfsA is recruited to the septum after actin ring constriction.

Because the *kfsA*-deletion strain showed abnormalities in conidiophores, we investigated the localization of KfsA in the conidiophore and found that KfsA localized at the septa between vesicle and metulae, and metulae and phialides (Fig. 7). In *S. cerevisiae*, Kin4 localizes predominately at the cortex of the mother cell. In midanaphase, Kin4 then localizes at the spindle pole body in the mother cell. We analyzed the localization of KfsA at spindle pole bodies

by using SNT18 strain (*alcA(p)::GFP::tubA, alcA(p)::kfsA::mRFP1*). In mitotic cells, we could not find any signal of KfsA-mRFP1 at the spindle pole bodies in both, hyphae and conidiophores (data not shown and Fig. 7).

If KfsA functions in checkpoint control during cytokinesis in *A. nidulans*, one would expect a loss of this control in the *kfsA*-deletion strain. We tested this possibility by using the replication inhibitor hydroxy urea (HU). The defects in chromosomal DNA metabolism induced by HU inhibit septum formation. It has been shown, that low level DNA damage (5 mM HU), nuclei divided in multiple rounds and septation were significantly delayed (Harris and Kraus, 1998). Under the same conditions, septum formation was not different in the *kfsA*-deletion strain (data not shown).

4. Discussion

In all eukaryotic cells cytokinesis has to be a well-controlled process to guarantee that each daughter cell receives a nucleus. The process is well characterized in *S. cerevisiae*, where the safeguard-machinery MEN (mitotic exit network) has evolved to prevent cytokinesis before proper segregation of the nuclei. One important component is the kinase Kin4, which inhibits the MEN and thus prevents precocious cell division when the spindle position checkpoint is activated. In filamentous fungi, such as *A. nidulans*, nuclear division and cytokinesis are not coupled in hyphae, resulting in multinucleate compartments. This changes during asexual spore formation at conidiophores. Metulae, phialides and spores are uninucleate and mitosis and cytokinesis are coupled processes. We characterized KfsA and found some functional similarities to Kin4 of *S. cerevisiae*. Deletion of the gene in *A. nidulans* caused a reduction in conidiospore production and an increase of septa in the conidiophore stalk.

Although sequence similarity of KfsA to Kin4 is rather low, the fact that the percentage of binucleate metulae increased in the *kfsA*-deletion strain and anucleate phialides were observed, points to similar functions of the two proteins. The number of 7% of binucleate metulae probably represents the number of metulae with a misaligned spindle. In wild-type KfsA would be transmitting this defect and prevent septation, but in the *kfsA* mutant septum formation occurs and traps the two nuclei in one cell. Another hint for a link of KfsA to septation is the localization of the protein to septa and an increased number of septa in conidiophore stalks. The latter observation suggests that KfsA normally suppresses septation in this cell structure. Assuming similar roles of KfsA and Kin4 indicates that in wild-type *A. nidulans* conidiophore stalks the MEN is active and an increase of the number of septa in the mutant would indicate defects in mitosis of some nuclei in the stalk. In wild type those defects would lead to the activation of the MEN and a suppression of septation, whereas in the *kfsA* mutant the regulatory network would not work and septa would form. In *S. cerevisiae*
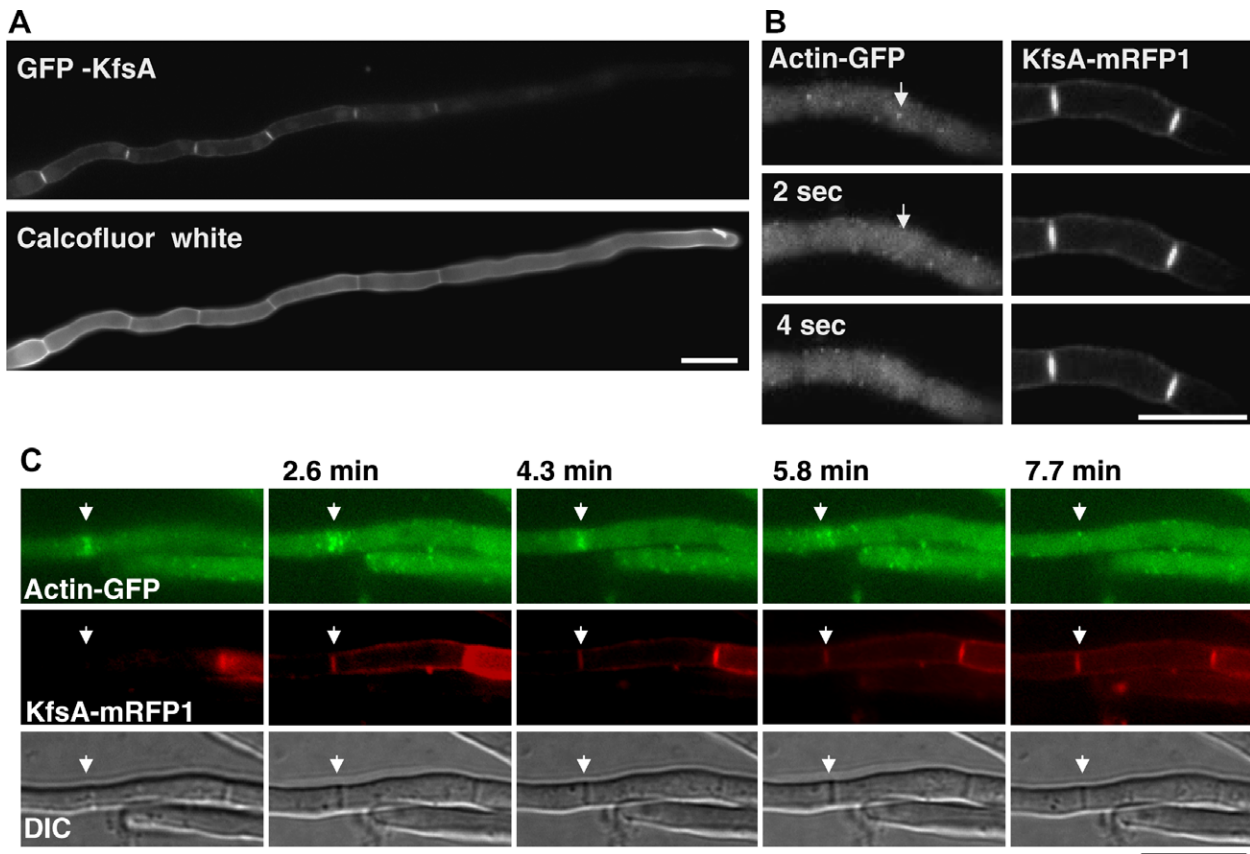
Fig. 6. KfsA localizes at septa and at the hyphal cortex. (A) Hypha of a GFP-KfsA expressing strain stained with calcofluor white and observed in the GFP channel and under UV light. (B,C) Time-lapse comparison of actin and KfsA localization at the hyphal cortex (B) and at septa (C) in SNT11 grown in liquid culture under non-repressing conditions. Actin and KfsA were mobile but actin-GFP spots were larger and easy to follow (arrows), whereas KfsA-mRFP1 spots were much smaller and movement was more difficult to see (compare movies in the supplementary files). Arrowheads indicate septation sites. Scale bar 10 μm.

the mitotic defect transmitted by Kin4 is a misalignment of the spindle. In *A. nidulans* stalks the number of nuclei can be about 100 and mitoses are synchronized. It seems that the spindles in the stalks are not aligned (Fischer and Timberlake, 1995). Whether this random orientation of the spindles is the reason for the activation of MEN and the lack of septa in stalks, is not known. An argument against this hypothesis is that in normal hyphal compartments spindles are also not aligned, but nevertheless septa are formed. Therefore, it could also be that KfsA senses other mitotic defects besides the position of the spindle. This is supported by the fact that we could not detect KfsA at spindle pole bodies. It is well known in *A. nidulans* that a DNA damage checkpoint exists, which controls septation (Wolkow et al., 1996,2000) and KfsA may be involved in this process. However, we did not find an effect of hydroxy urea on septation in the *kfsA*-deletion strain different from wild type. In addition, it is unlikely, that from 100 nuclei found in the stalk of a conidiophore, always some have a mitotic defect. Therefore, it can also be that the suppression of septation in conidiophores depends on a regulatory network independent of mitosis and that this is defect in the *kfsA* mutant.

A similar phenotype to that of the *kfsA*-deletion strain was reported for a mutation in the cyclin-dependent kinase NimX (Ye et al., 1999). Mutation of NimX to a non-phosphorable version led to drastic developmental phenotypes. Conidiophores were highly abnormal and, similar to the *kfsA* mutation, septated. However, *kfsA* mutant strains did not show any abnormal conidiophores but only a reduction in the number of spores and an increase of the number of septa. This makes it unlikely that NimX is a direct target for KfsA.

The discussed roles for KfsA suggest an early action of the protein during septation. However, in the time-dependent localization experiment KfsA appeared after the contractile actin ring was formed and remained at mature septa. This indicates additional roles in septum formation itself, after the function of e.g. *sepH*, which is essential for actin ring assembly (Bruno et al., 2001) and localization of the septin protein AspB and the formin-related SepA (Harris et al., 1997; Momany and Hamer, 1997; Sharpless and Harris, 2002; Westfall and Momany, 2002). It should be noted that in filamentous fungi septa are not completely closed, but a cytoplasmic bridge through a central pore connects two neighbouring compartments. In addition,
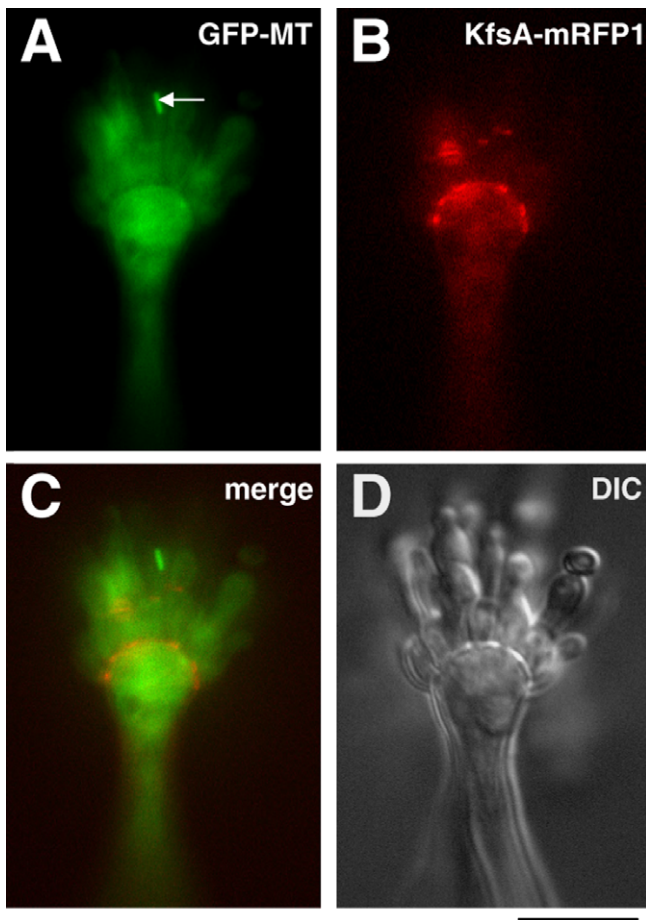
Fig. 7. KfsA-GFP localizes at the septa between vesicle and metulae and metulae and phialides. Strain SNT18 was grown for three days at 30 °C on microscope slides covered with MM + 2% Glycerol + 0.8% agarose. The arrow in (A) indicates a mitotic spindle. Scale bar 10 μm.

the septum is not a "dead" structure, but organelles are associated to it. Well characterized are the Woronin bodies, peroxisomal derivatives with a protein crystal inside, which plug the septal pore upon injuries of the hyphae (Jedd and Chua, 2000). Furthermore, there is good evidence that active microtubule organizing centres are associated to the septa (Veith et al., 2005). The complexity of the septum in filamentous fungi suggests a more complex and continuous regulation than in yeasts and KfsA might play a role there. The identification of KfsA-interacting proteins and other studies should help to further unravel the function of this interesting putative kinase protein.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2007.03.006.

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