Molecular Microbiology (2006) **61**(2), 544–554
doi:10.1111/j.1365-2958.2006.05257.x
First published online 15 June 2006

The $Zn(II)_2Cys_6$ putative transcription factor NosA controls fruiting body formation in *Aspergillus nidulans*

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Summary

The filamentous fungus *Aspergillus nidulans* reproduces asexually with conidiospores and sexually with ascospores, both of which are the result of complex morphogenetic pathways. The developmental decisions for both ways of reproduction largely depend on the action of stage-specific transcription factors. Here we have characterized the putative $Zn(II)_2Cys_6$ transcription factor NosA (number of sexual spores), a protein of 675 aa, which shares 44% sequence identity to Pro1 from *Sordaria macrospora* and 43% identity to *A. nidulans* RosA, a second protein of that class. The *nosA* gene was constitutively expressed during the life cycle of *A. nidulans* and was upregulated during late asexual development and upon carbon starvation. The NosA protein localized to nuclei. Both, NosA and RosA, regulate sexual development. Whereas RosA plays a role in early decisions and represses sexual development, NosA activity is required for primordium maturation. Interestingly, the two factors are genetically linked, because RosA repressed NosA expression. This illustrates that the balance of these two $Zn(II)_2Cys_6$ proteins determines the fate of vegetative hyphae to undergo sexual development.

Introduction

Soil-borne microorganisms regularly face the problem of changing environmental conditions in their natural habitat and hence have evolved mechanisms to cope with those challenges. Filamentous fungi commonly react on changing conditions with the production of spores (Fischer and Kües, 2003). The ascomycete *Aspergillus nidulans* is able to form asexual and sexual spores, both of which have distinct properties and are produced under different conditions. Whereas asexual spores serve as units to spread in the environment, sexual spores are generated to persist harsh conditions. Both developmental programs are tightly regulated and involve a high number of specific genes. Among them are those, which encode proteins involved in signal perception and signal transduction as well as the ones directly required for the morphological and physiological changes (Adams *et al.*, 1998).

In *A. nidulans* sexual reproduction starts with the fusion of two haploid hyphae. These can be hyphae of the same mycelium or of a different one, because *A. nidulans* is a homothallic fungus and the genome harbours both mating genes (Galagan *et al.*, 2005). The first sign of sexual development is the formation of Hülle cells, thick-walled cells, which later surround the mature fruiting bodies. Embedded into those nests of Hülle cells, young fruiting bodies, primordia, are produced. Within those primordia nuclear fusion and formation of diploid nuclei occurs in the ascogenous hyphae, where subsequently meiosis reduces the chromosome number again to the haploid stage. Meiosis is followed by one mitosis. Each ascus hence contains eight haploid ascospores, which subsequently become binucleate. Thousands of asci develop in one fruiting body, which is surrounded by a melanized shell of dead cells. The knowledge of the molecular biology underlying the developmental pathway is rather limited. In principle, signalling may require components at the cell membrane, in the cytoplasm and in the nucleus. Corresponding signalling components and a number of differentially expressed genes have been characterized in the past few years in *A. nidulans* (Fischer and Kües, 2006).

In a systematic approach several membrane-bound G-protein coupled receptors (nine in the genome) have been characterized, three of which are involved in the regulation of development (Han *et al.*, 2004; Seo *et al.*, 2004). When either *gprA* or *gprB* were deleted, *A. nidulans* only produced few, small cleistothecia and double mutants were unable to undergo sexual development at all. This phenotype occurred only during self-fertilization, while outcrossed strains behaved normal, suggesting a specific role for GprA and GprB in homothallic reproduction. Overexpression of the transcription fac-

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tor NsdD (never sexual development) could partially restore the capability of sexual reproduction and the authors concluded that GprA and B are likely to activate additional genes necessary for the completion of the process. In comparison, lack of GprD caused pleiotropic phenotypes and appeared to be involved in different pathways. Colonies were very small and covered by cleistothecia. From this the authors concluded that GprD represses sexual development in the wild type. Signalling from G-protein coupled receptors occurs, as the name suggests, through heterotrimeric G-proteins. In *A. nidulans* a G-protein, FadA, has been characterized as a central regulator for the balance between vegetative growth and development (Lee and Adams, 1994; Yu *et al*., 1996; Rosén *et al*., 1999). However, genetic data indicated that this G-protein is probably not involved in signalling of GprA, B or D. In addition to the G-protein alpha-subunit FadA, two other alpha-subunits have been identified in the genome of *A. nidulans*, GanA and GanB (Chang *et al*., 2004; Lafon *et al*., 2005). They appear to play a role in carbon sensing and germination rather than in sexual development. Hence, it remains open how the GPCRs transmit their signals in the cell. Recently, a membrane oxidoreductase involved in the synthesis of a developmental signal was characterized (Soid-Raggi *et al*., 2006). This signalling component appears to play a specific role in regulation of asexual development.

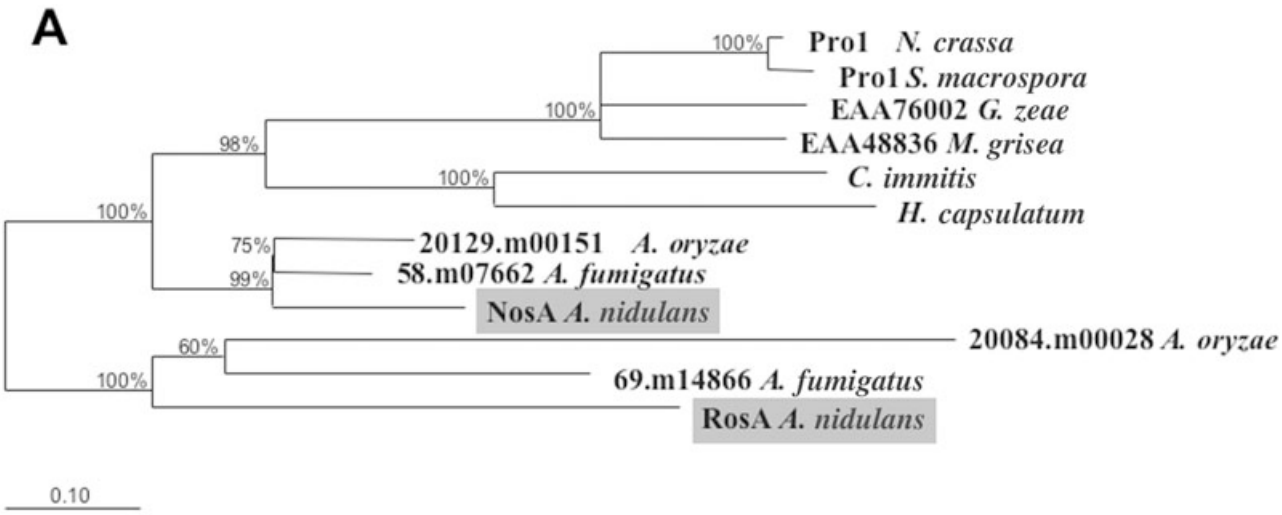
Signalling cascades in the cytoplasm comprise for instance the cAMP pathway and the MAP kinase cascade (Fillinger *et al*., 2002; Wei *et al*., 2003). The latter appears to play a specific role in the regulation of sexual development. Deletion mutants of the MAP kinase kinase kinase, SteC, affected hyphal fusion and fruiting body development (Wei *et al*., 2003).

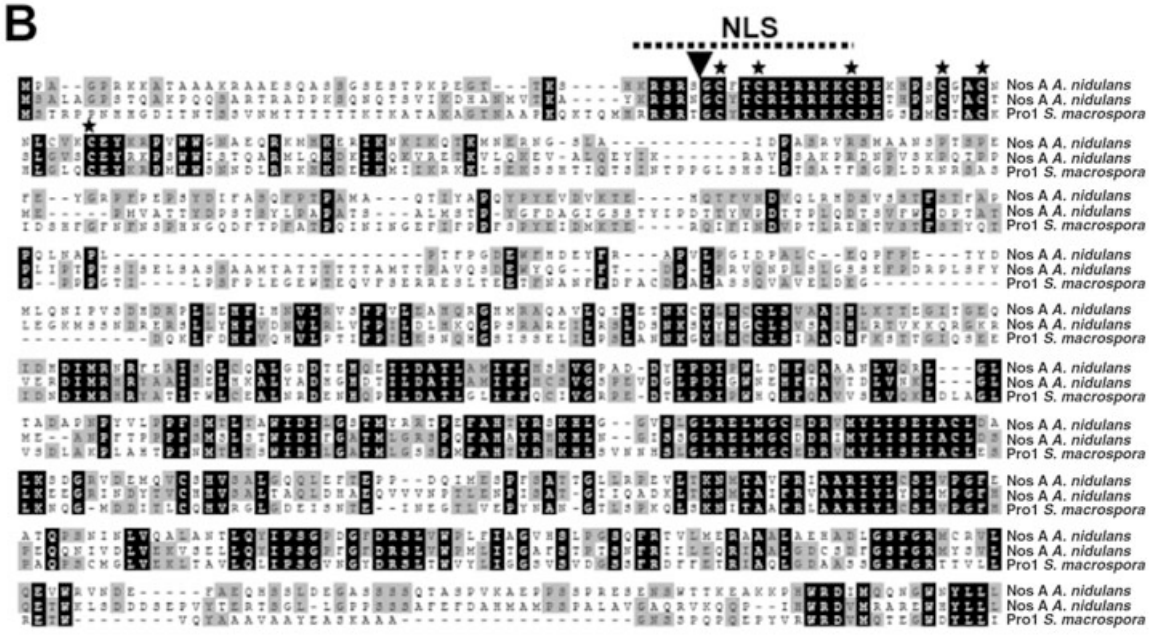
The direct or indirect targets of the described signalling pathways should be transcription factors, which lead to the activation or repression of specific genes. A number of those genes have been described in the past, among which are the activators NsdD and StuA as well as repressors such as RosA and StuA (Miller *et al*., 1992; Han *et al*., 2001; Vienken *et al*., 2005). The latter factor was isolated due to its homology to the $Zn(II)_2Cys_6$ class activator, Pro1, for perithecium development in *Sordaria macrospora* (Masloff *et al*., 1999; Masloff *et al*., 2002). This class of transcription factors is the dominant class of regulators in fungi with more than 100 members in *A. nidulans* and *Neurospora crassa* (Todd and Andrianopoulos, 1997; Borkovich *et al*., 2004; Galagan *et al*., 2005). In contrast to the effect of *pro1* mutation in *S. macrospora*, lack of RosA in *A. nidulans* caused an induction of sexual development rather than a block of it. Therefore, it was concluded, that RosA acts as a repressor (Vienken *et al*., 2005). In this article we describe a second homologue of Pro1, named NosA (number of sexual spores), and show that it plays an activating function in fruiting body development.

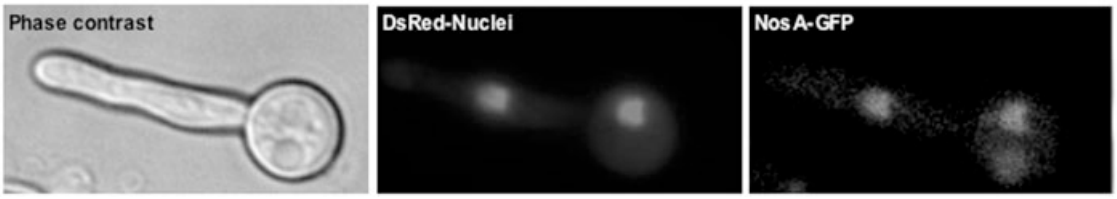
Results

Aspergilli contain two Pro1 homologues

In order to analyse the role of putative orthologues of the sexual developmental regulator Pro1 from *S. macrospora* in *A. nidulans*, we analysed the genome databases at the Whitehead Institute (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/) (Galagan *et al*., 2005). Interestingly, we found two proteins with high similarity to Pro1, designated RosA and NosA (Fig. 1). RosA was described previously as a repressor of sexual development (Vienken *et al*., 2005). In this study, we have characterized the second gene, *nosA*. The genomic sequence of *nosA,* as deposited in the database under the Accession no. AM231027, was confirmed by polymerase chain reaction (PCR) amplification of small fragments and sequencing (results not shown). To determine the intron–exon borders, we amplified corresponding cDNAs by reverse transcription (RT)-PCR. Comparison of genomic and cDNA sequences revealed the presence of two introns (62 and 67 bp) in the 5′-region of the gene. The position of the first intron is conserved in the *rosA* gene locus and also in orthologues of *S. macrospora* (Fig. 1A), *Sordaria brevicollis* and *N. crassa* (data not shown). The predicted NosA protein comprises 675 amino acids and has a predicted molecular mass of 75.6 kDa and a calculated isoelectric point of 6.3. Sequence identity between the predicted proteins NosA and RosA was about 43%, between NosA and Pro1 from *S. macrospora* 44%. The identity between RosA and Pro1 was 38%, which is slightly lower than between NosA and Pro1. Interestingly, only the genomes of species in the group of the Aspergilli (*Aspergillus oryzae* and *Aspergillus fumigatus*) harboured two proteins with similarity to Pro1. Other ascomycetes, such as *N. crassa*, *Podospora anserina*, *Magnaporthe grisea* and *Histoplasma capsulatum* (genome sequence not complete yet) had just one homologue, which showed more identity to NosA (Fig. 1). There were no significant hits in the genomes of *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans, Ashbya gossypii* or the basidiomycetous fungi *Cryptococcus neoformans*, *Ustilago maydis* and *Coprinus cinereus* (not shown). The fact that *A. oryzae* and *A. fumigatus* contained homologues of RosA and NosA was at the fist glance surprising, considering that they are asexual species. However, meanwhile a systematic genome-wide approach revealed that a large number of genes, involved in sexual development in *A. nidulans* or other sexual fungi, are conserved among *A. oryzae* and *A. fumigatus,* suggesting the potential of a







sexual cycle in these fungi (Dyer *et al*., 2003; Galagan *et al*., 2005; Nierman *et al*., 2005).

NosA is a nuclear protein

Protein sequence analysis of NosA revealed a $Zn(II)_2Cys_6$ binuclear cluster domain in the region from 44 amino acids downstream of the ATG to amino acid 74. Overlapping with the binuclear cluster domain, we found a bipartite nuclear targeting sequence from amino acid 39–55 (Fig. 1A) (pattern tools at http://www.expasy.org). To test the functionality of the nuclear localization site (NLS), we fused the reporter-gene *sGFP* to the C-terminus of the *nosA*-ORF and put the construct

Fig. 1. Sequence analysis of putative Pro1-like proteins and subcellular localization of NosA.
A. NosA from *A. nidulans* shows higher similarity to the Pro1 sequences. The tree was calculated with the ARB program (Ludwig *et al*., 2004) using distance and position-based algorithm with different evolutionary models. The 0.1 scale bar shows 10% sequence aberration. The numbers at the branch points represent the percentage with which the same point was calculated in 100 independent calculations. The numbers at the species names indicate the accession numbers of the proteins in the corresponding databases.
B. Alignment of NosA (AM231027) with Pro1 from *S. macrospora* (Accession no. Q9UVG3) and *A. nidulans* RosA (Accession no. CAD58393). If amino acids were identical in two sequences, they were shaded in grey and if they matched in all three proteins, they were shaded in black. The alignment was done with DNAStar using Megalign (Clustal) with a gap penalty and a gap length penalty of 10. The cysteine residues involved in the coordination of the Zn atoms are highlighted with an asterisk above the sequences. The position of a conserved intron is indicated by an arrow head. The predicted bipartite nuclear localization signal (NLS) is indicated with a dashed line above the sequence.
C. Intracellular localization of NosA-GFP in nuclei. The fusion protein was expressed under the control of the *alcA* promoter and grown for 10 h at 37° in medium with 2% ethanol as carbon source. Nuclei were visualized with nuclear-targeted dsRedT4 (Toews *et al*., 2004).

under the control of the inducible alcohol dehydrogenase promoter *alcA*. The construct was transformed into the *A. nidulans* wild-type strain SRF200 and germlings analysed for GFP fluorescence. All germlings showed spot-like GFP-distribution under inducing conditions (ethanol as carbon source). The spots were proven to be nuclei by costaining with a nuclear-targeted dsRedT4 protein (Fig. 1C). This was in contrast to the localization pattern of RosA, which was found predominantly in the cytoplasm (Vienken *et al*., 2005). However, because truncated versions of RosA were able to translocate to the nucleus, we suggested that the protein potentially enters the nucleus, perhaps after signalling-dependent modification.

nosA *is upregulated during late asexual development and upon carbon starvation*

As a first approach to study the molecular role of NosA, we studied the expression of the gene during the life cycle of *A. nidulans* (strain FGSC4). Northern-blot analysis revealed low constitutive expression, with an increase of the steady-state level of *nosA* transcript at the late stages of asexual development (Fig. 2). Because surface cultures of *A. nidulans* may be glucose-limited during late asexual development, we tested whether starvation had an effect on *nosA* expression. Indeed, under those conditions (liquid culture grown for 16 h, then 3 h in MM without a carbon source) strong *nosA* expression was observed. Accumulation of *nosA* mRNA also appeared when a liquid culture was grown for 48 h or longer (Fig. 2).

The nosA *gene is required to complete the sexual cycle*

As a next step to explore the function of *nosA* in *A. nidulans,* we deleted the entire open reading frame from the genome and replaced it with the *A. nidulans* auxotrophic marker *argB* (for details see *Experimental procedures*). The deletion was performed in the *veA1* mutant strain SRF200 and confirmed via PCR and Southern blot (Fig. 3). Among 60 transformants only one strain (SKV31) harboured the correct gene deletion arrangement in the genome. Colony growth of SKV31 was equivalent to wild type and asexual development appeared to be normal. In contrast, the deletion strain was unable to undergo sexual development even under conditions, where this pathway is favoured in wild-type strains (2 weeks incubation, increased CO_2 concentration, dark incubation) (data not shown). Because *A. nidulans* is a homothallic fungus, a mating partner is not required. We named the gene *nosA*. To make sure

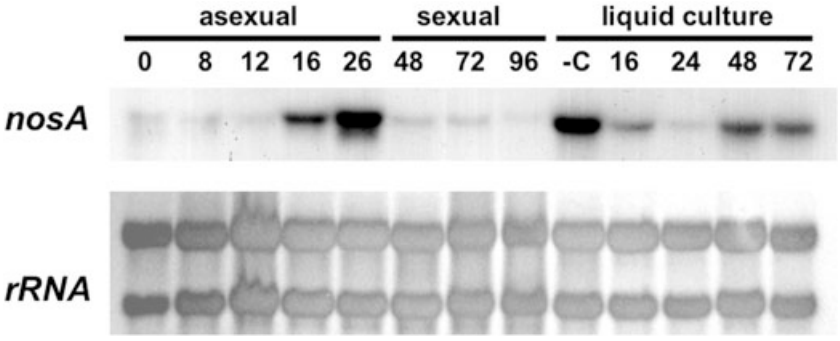


Fig. 2. Transient expression of *nosA* during development and upon starvation. *A. nidulans* FGSC4 was induced for differentiation and mycelium harvested at the time points indicated. See *Experimental procedures* for details. In addition, mycelia grown in liquid culture was harvested after 20 h, washed and transferred into medium lacking any carbon source for 2 h. RNA was isolated of all samples and 15 μg processed for Northern blots using a 800 bp ^{32}P-labelled *nosA*-specific probe of the open reading frame generated by PCR. As a loading control we stained the ribosomal RNA after transfer to the membrane with methylene blue. Transcript was detectable in all stages, but was upregulated in late asexual development and under starvation conditions.

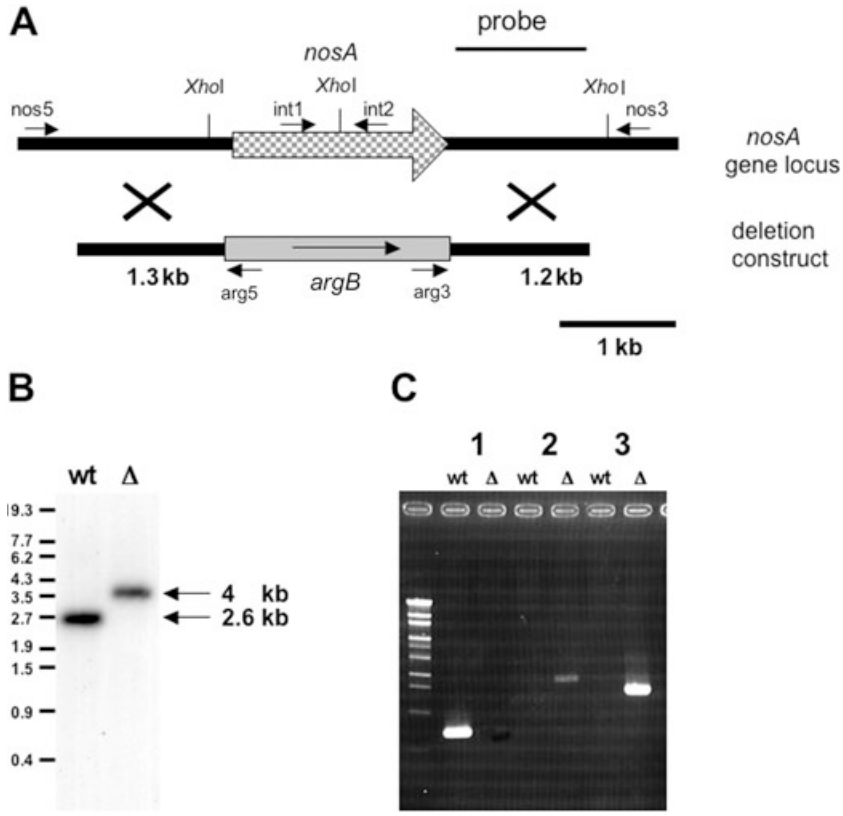


Fig. 3. Deletion of the *nosA* gene.
A. Scheme of the deletion construct and the genomic region.
B. Southern blot analysis of a *nosA*-deletion (Δ; SKV31) and a wild-type (wt; SRF200) strain. Genomic DNA was isolated, restricted with XhoI, separated on a 1% agarose gel, blotted and hybridized with the ^{32}P-labelled probe indicated in A.
C. Demonstration of the *nosA*-deletion event via PCR analysis using three oligonucleotide pairs. 1: int1 and int2; 2: nos5 and arg5; 3: nos3 and arg3. Positions of the oligonucleotides are indicated in A. PCR fragments were separated on a 1% agarose gel and stained with ethidium bromide.

that SKV31 did not contain any other mutations, we crossed it to an *argB*-auxotrophic strain (RMS011) and selected strains from the progeny with the *nosA*-deletion event. The Δ*nosA*-phenotype cosegregated with the *argB*-marker and Southern Blot analysis confirmed the deletion event in *argB*$^+$ strains (four), and the wild-type situation in *argB*$^-$ strains (four). This experiment did not only show linkage of the phenotype with the nutritional marker, but also demonstrated that the complete sexual cycle was possible when the *nosA*-deletion strain was crossed to a wild type. This suggests that *nosA* mutation is recessive.

Because common laboratory strains, such as SRF200 or RMS011, harbour a mutation in the developmental regulator *veA*, we tested the effect of the *nosA* deletion in a *veA*$^+$ background (Fig. 4). We crossed SKV31 with WIM126 and selected a *ΔnosA, veA*$^+$ strain (SKV32) and compared it to the *nosA*$^+$*, veA*$^+$ strain FGSC4. The deletion of *nosA* was confirmed by Southern blotting and the presence of the *veA*$^+$ allele was confirmed by sequence analysis of the corresponding part of the gene (data not shown). We incubated the strains in the presence and absence of light, because *veA* is involved in light-dependent development (Mooney and Yager, 1990). In light, both strains developed asexually whereas under dark conditions, FGSC4 reproduced mainly sexually, and SKV32 initiated the sexual cycle but development was blocked at the primordial stage. We could not identify any differentiated cells, such as hooks or ascus mother cells. Hülle cells were hardly found. Occasionally very small cleistothecia (about 30 μm diameter instead of 300 μm) were produced. Because *nosA* deletion was achieved with argB as marker, we tested the developmental phenotype also on agar plates supplemented with arginine to account for the possibility that the *argB* gene was not fully functional and caused the observed phenotypes. However, also under these conditions the strain displayed the same developmental block. Despite the small size of the cleistothecia they developed fertile ascospores. The number of ascospores per cleistothecium was however, reduced to less than 100 in comparison to 10^4–10^5 in wild-type cleistothecia.

The availability of several *nosA*-progeny strains with different genetic markers allowed also testing for mating between two *nosA*-deletion strains (SKV31 and SKV38). Heterokaryon formation occurred like in wild type, suggesting that hyphal fusion and heterokaryon maintenance were not impaired, but only primordia and some microcleistothecia were observed (data not shown).

Interaction of nosA *with other developmental regulators*

In order to determine which genes act upstream of the putative transcription factor NosA, we studied the relation-

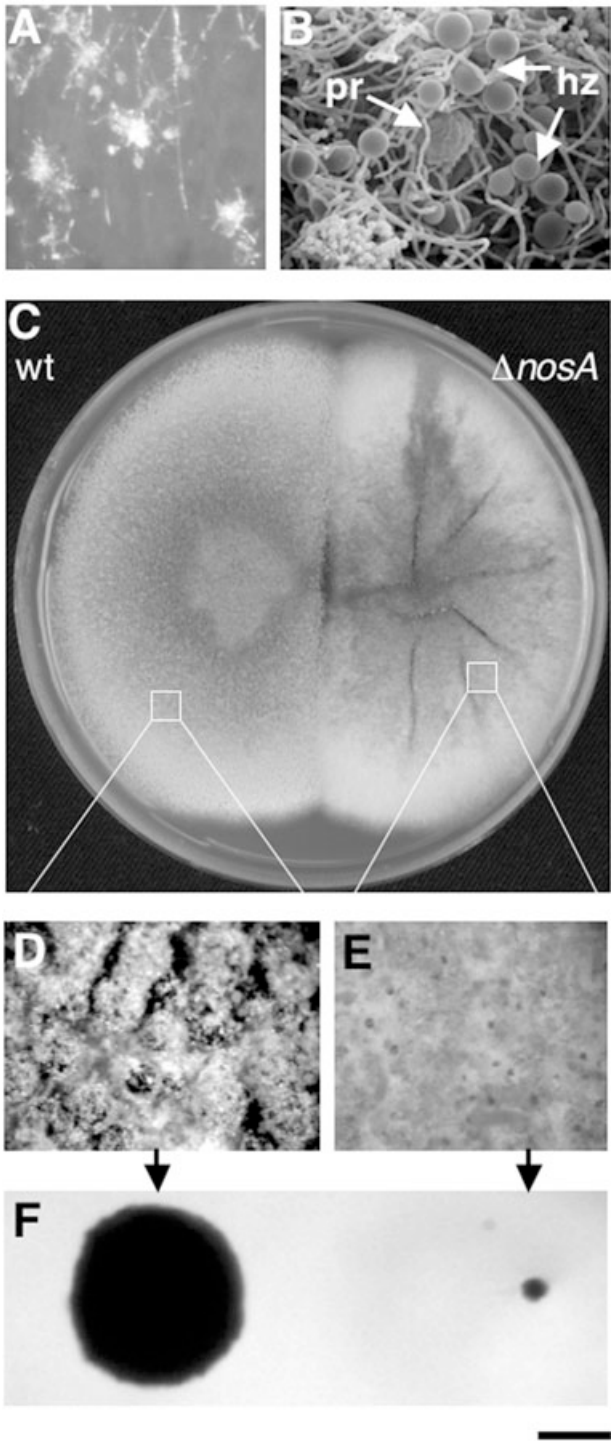


Fig. 4. Phenotype of a *nosA*-deletion strain in comparison to wild type.
A and B. In a *ΔnosA, veA*$^+$ strain (SKV32) fruiting body formation usually stops at the primordium (pr) stage, where the primordium is surrounded by Hülle cells (hz). (A) Low magnification in the light microscope. (B) Scanning electron microscope picture.
C. *ΔnosA* strain (SKV32) and wild-type FGSC4, both *veA*$^+$, point-inoculated and grown for 5 days at 37° under conditions promoting sexual development.
D and E. In wild type large numbers of mature fruiting bodies are found (D), whereas in the *ΔnosA* strain only some microcleistothecia are visible (E).
F. Size comparison of a wild-type and a *ΔnosA* fruiting body. The scale bar represents 100 μm in A and F, 50 μm in B, about 1 cm in C and 500 μm in D and E.

ship to *nsdD* because corresponding deletion strains fail to initiate sexual reproduction and hence appear to be blocked before the proposed action of NosA. The *nsdD* gene encodes a GATA factor-like transcription factor, which is essential for sexual development (Han *et al.*, 2001). Likewise, overexpression of the gene forces cleistothecium formation even under conditions, where wild-type strains do not form cleistothecia. We constitutively expressed *nsdD* under the control of the *gpdA*-promoter in a *nosA*-deletion strain and found that only primordia occurred (data not shown). This suggests that *nosA* acts in the same pathway downstream of *nsdD* or in a parallel pathway to *nsdD*.

In a previous publication we showed that expression of *nsdD* in liquid culture is repressed by another regulator of the $Zn(II)_2Cys_6$ class transcription factors, RosA (Vienken *et al.*, 2005). Deletion of *rosA* resulted in a transcriptional upregulation of *nsdD* and to the induction of early sexual developmental structures (Hülle cells). To test the possibility that RosA regulates *nosA* in a similar way, we analysed *nosA*-transcript levels in a wild-type and in a *rosA*-deletion strain (Fig. 5). In wild type the *nosA* transcript was upregulated after 72 h of growth in liquid culture whereas in the *rosA*-deletion strain the expression was much stronger in all stages. These results suggest that RosA represses, directly or indirectly, the expression of *nosA*.

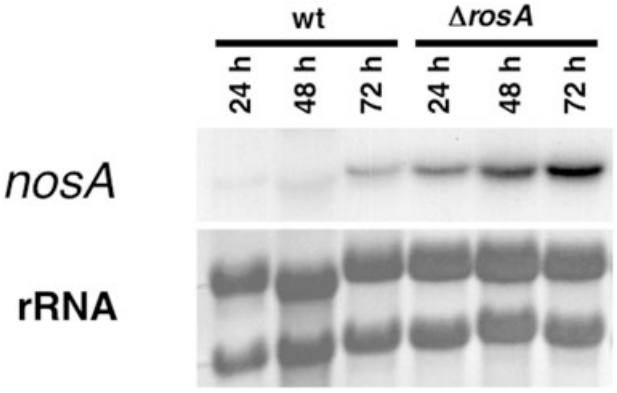


Fig. 5. Northern blot analysis of *nosA* in a wild-type and a *ΔrosA* strain during growth in submersed culture. Cultures of wild type (wt = FGSC26) and mutant (Δ*rosA* = SKV8) were harvested after 24, 48 and 72 h, RNA isolated and subjected to Northern blotting. The membranes were hybridized with a *nosA* gene-specific DNA fragment labelled with ^{32}P. Prior to hybridization, rRNA was stained with methylene blue as loading control.

We have characterized several genes differentially expressed during sexual development in the past and tested now, whether they are under the control of *nosA*. To this end, we compared the expression of catalase-peroxidase (*cpeA*) and a high-affinity glucose transporter, *hxtA*. These two genes are strongly expressed during sexual development and also under carbon starvation conditions. Because *nosA*-deletion strains are strongly impaired in sexual development, we analysed *cpeA* and *hxtA* expression only under starvation conditions. For both genes, a drastic decrease of the transcript level was observed in *nosA*-deletion strains. However, *cpeA* transcript was still detectable in the absence of *nosA*, suggesting another factor controlling its expression. The result for *hxtA* was confirmed with a promoter fusion with GFP. Whereas GFP fluorescence was observed in wild-type starving cells, no fluorescence was obtained in the *nosA*-deletion strain (Fig. 6).

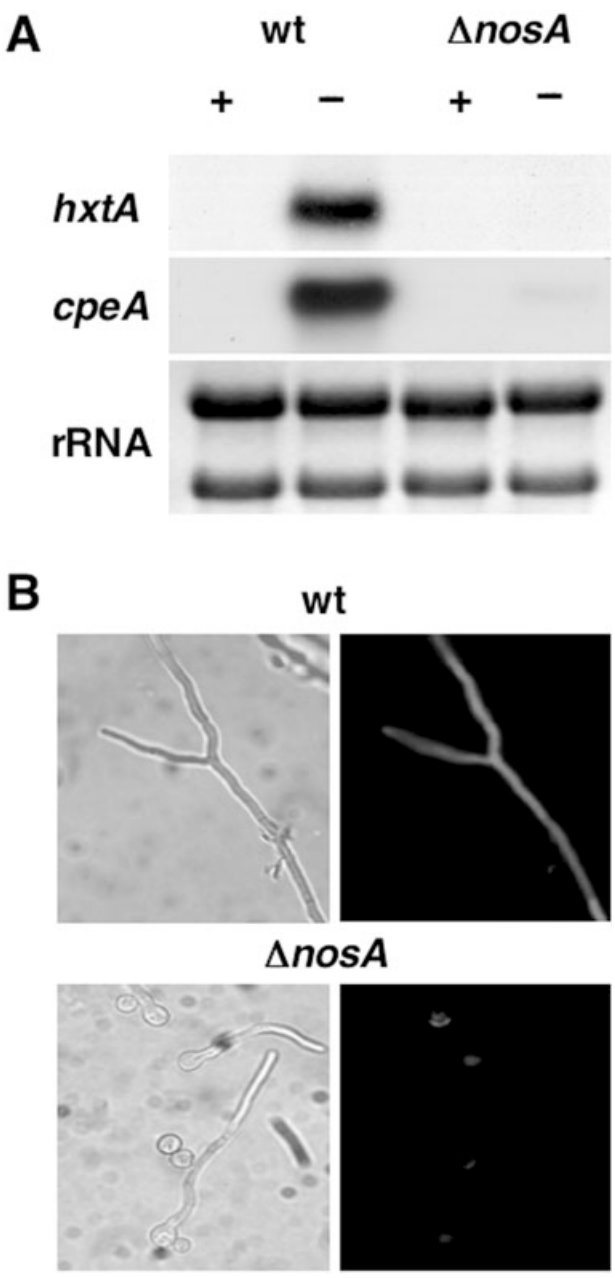


Fig. 6. Transcriptional regulation of two genes expressed during sexual development, *hxtA* and *cpeA*, by *nosA*.
A. Northern-blot analysis of vegetative growing myceliums with (+) and without (–) glucose. Starvation induced *hxtA* expression in wild type (wt; FGSC26) but not in the *ΔnosA* strain (SKV31). *cpeA* was strongly upregulated in wild type but only very weakly expressed in the *ΔnosA* strain.
B. Promoter–GFP fusion of *hxtA*. To confirm the results of the Northern-blot analysis plasmid pHHgfp4 (*hxtA*(*p*)*:sgfp*) was transformed into SRF200 (wild type) and SKV31. Under starvation conditions a bright GFP signal was detectable in wild type, but not in any of 20 examined transformed *ΔnosA* strains. The weak signals in the *ΔnosA* strain were due to autofluorescence of spores.

Discussion

Sexual fruiting body development in fungi is the result of the integration of a number of environmental signals, such as light, pheromones or the nutritional status. These signals are sensed by the fungus and finally lead to differential gene expression. Many signalling cascades, which are involved in fungal development, are principally conserved among fungi (Lengeler *et al*., 2000). The specificity is largely due to transcription factors, which are stage-specifically expressed or modulated in their activity in response to the activation of the signalling cascades. A number of different transcription factors or regulators have been described in *A. nidulans*, many of which play a role early during sexual development. Such genes are for instance *nsdD* or *veA* (Mooney and Yager, 1990; Han *et al*., 2001; Kim *et al*., 2002). Later stages of cleistothecium development are only poorly understood at the molecular level. In this study we used a reverse genetic approach to analyse the role of a homologue of the Pro1 transcription factor from *S. macrospora*. Pro1 was identified in a mutant screening for strains impaired in perithecium formation. The protein encodes a $Zn(II)_2Cys_6$ transcription factor, which is required for protoperithecium maturation (Masloff *et al*., 1999). Deletion analysis of the homologue, NosA, revealed that NosA might play a similar role in *A. nidulans*. *nosA*-mutant strains generally failed to develop mature fruiting bodies and development was blocked at the primordial stage. The switch between vegetative and sexual development was – in contrast to previously described factors – unaffected. However, it has to be noted, that the original *nosA* mutant as a *veA1/ΔnosA* double mutant did not produce cleistothecia. Because *veA1* mutants only poorly develop sexually, we anticipate that the complete lack of cleistothecia in the double mutant

does not indicate a specific role for NosA at the initiation of sexual development. NosA thus is the first regulator described for an intermediate developmental step of cleistothecium formation in *A. nidulans*. However, some mature cleistothecia were observed. This result indicates that in some instances the block can be overcome, the entire developmental program completed and fertile ascospores produced. This is also the case for NsdD (Han *et al*., 2001). Although the name *never sexual development* suggests a complete failure to develop sexual structures, occasionally cleistothecia of normal size are observed. It has to be noted, that cleistothecia in Δ*nosA*-strains were only very small in comparison to wild-type cleistothecia. The phenomenon of microcleistothecia was described before for mutants with defects in amino acid metabolism. This is true for mutations in the biosynthetic pathway as well as for mutations in regulators of the cross-pathway control (Eckert *et al*., 1999; Hoffmann *et al*., 2000). Therefore, the failure to develop normal-size cleistothecia in Δ*nosA* strains could be due to nutritional limitation. However, this would not explain why the block at the primordial stage can be overcome in some cases. A similar phenotype of small cleistothecia was observed in mutants of the GPCRs GprA and B (Seo *et al*., 2004). This could indicate that GprA and/or GprB and NosA are components in the same developmental pathway and that NosA acts downstream of the GPRCs. An argument against this hypothesis is that overexpression of *nsdD* in a Δ*gprA*/Δ*gprB* double mutant did partially restore the ability to produce cleistothecia. If NosA activity would be required downstream of GPRCs, cleistothecia development should have always stopped at the primordium stage in the overexpression experiment. That was not the case. In addition, overexpression of *nsdD* in Δ*nosA* could not overcome the *nosA* block. Other mutations, which result in a developmental block at the primordium stage are mutations in components of the COP9 signalosome (Busch *et al*., 2003). As a difference to the effect of the *nosA* deletion, COP9 mutants produce primordia constitutively independent of light. This suggests an early role of COP9 as well.

Several other positive regulators of sexual development were characterized by their potential to induce sexual differentiation upon overexpression (Han *et al*., 2001; Kim *et al*., 2002). Likewise, the lack of a negative regulator had a similar effect (Vienken *et al*., 2005). In contrast, overexpression of *nosA* (induction with ethanol) did not cause any developmental phenotype (data not shown). This might be explained by the fact that VeA, NsdD and RosA act early in development and thus are likely to control the activity of a larger number of genes, which together induce the process. In contrast, NosA acts at a later stage and thus overexpression in liquid culture probably activates genes required for primordium maturation but not for the initiation of the sexual cycle. The fact that overexpression of NsdD did not induce sexual development in Δ*nosA* strains, illustrates this nicely.

If NosA regulates genes necessary for maturation of primordia, the question is how this is achieved. In asexual development several transcription factors are stage-specifically expressed (Mirabito *et al*., 1989; Adams *et al*., 1998). This seems not to be the case for *nosA*, because the transcript was detectable in all stages of the life cycle. This is also true for other regulators of sexual development, e.g. *nsdD, rosA* or *stuA*. This suggests that the activity of those regulators is likely to be regulated at a post-transcriptional level and might reflect the large number of factors which are integrated to induce sexual development. We do not have any experimental evidence yet for such a regulation of NosA activity. Another explanation for the constitutive expression is that those regulators are also involved in other processes. Likewise, *nosA* was transcriptionally upregulated upon starvation and during late asexual development. The latter condition is probably also characterized by starvation, because the conidiophores are growing into the air and they might be nutrient-limited. The fact that NosA appeared to regulate the expression of the high-affinity hexose transporter, *hxtA* and the catalase-peroxidase, *cpeA*, both of which are normally induced upon nutrient limitation, suggests a positive role of NosA during starvation (Scherer *et al*., 2002; Wei *et al*., 2004).

NosA is the third $Zn(II)_2Cys_6$ protein characterized in *A. nidulans* with a role in developmental regulation, RosA, OefC and NosA (Lee *et al*., 2005; Vienken *et al*., 2005). Whereas RosA plays a role in early regulation and mainly represses sexual development, NosA presence is required for cleistothecium maturation. Interestingly, the two factors are genetically linked, because RosA represses NosA expression. The availability of the *A. nidulans* genome sequence opens now the possibility to apply genome-wide approaches, such as transcriptional profiling or proteomics, to further understand *A. nidulans* development (Galagan *et al*., 2005; Nowrousian *et al*., 2005). The *nosA* mutant is especially useful for these approaches because it regulates primordium maturation without affecting hyphal growth or asexual reproduction.

Experimental procedures

Strains, plasmids and culture conditions

Supplemented minimal and complete media for *A. nidulans* were prepared as described, and standard strain construction procedures were used (Hill and Käfer, 2001). To isolate asexual development-specific RNA, corresponding strains were pre-grown in liquid culture for 16 h and the mycelium subsequently filtered (miracloth) and the filters transferred to solid medium. For sexual development-specific RNA conidiospores (10^5) were plated onto cellophane membranes

Table 1. *A. nidulans* strains used in this study.

Strain	Genotype	Source
FGSC26	*biA1; veA1*	FGSC, Kansas, USA
FGSC4	Wild type	FGSC, Kansas, USA
SRF200	*pyrG89; ΔargB::trpCΔB; pyroA4; veA1*	Karos and Fischer (1999)
RMS011	*pabaA1, yA2; ΔargB::trpCΔB; veA1*	Stringer *et al.* (1991)
WIM126	*pabaA1, yA2; veA*$^+$	Champe and Simon (1992)
KHH52	*pabaA1, yA2; ΔargB::trpCΔB; ΔnsdD::argB; trpC801, veA*$^+$	Han *et al.* (2001)
SWHHgfp	SRF200 transformed with pHHgfp4; *ΔargB::trpCΔB; pyroA4; veA1; hxtA(p)::sGFP*	Wei *et al.* (2004)
SKV8	Deletion of *rosA* in SRF200; *pyrG89; ΔargB::trpCΔB; pyroA4; veA1; ΔrosA::argB*	This study
SKV31	Deletion of *nosA* in SRF200; *pyrG89; ΔargB::trpCΔB; pyroA4; veA1; ΔnosA::argB*	This study
SKV32	Cross between SKV-31 × WIM126; *veA*$^+$*ΔnosA::argB*	This study
SKV37	Cross between SKV-31 × WIM126; *pyrG89; veA*$^+$*ΔnosA::argB*	This study
SKV38	*pabaA1, pyrG89; yA2; veA*$^+$*ΔnosA::argB*	This study
SKV50	Cross between KHH52 and SRF200; no marker; *ΔnsdD, veA*$^+$	This study
SKV103	Wild type; cross between WIM126 × SRF200; *pyrG89; pyroA4; veA*$^+$	This study

(Gehring, Bielefeld) on solid medium. Mycelium was harvested at given time points and immediately processed for RNA isolation (see below). 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.

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 and RNA analyses (Southern and northern hybridizations) were performed as described by Sambrook and Russel (1999).

Deletion of nosA

The *nosA* flanking regions were amplified by PCR using genomic DNA and the primers NosA5 (5′-TCG GTACATCTAGGACAGCC-3′) and NosA5-SfiI (5′-TGG TGGCCATCTAGGCCTGTAATAAACGGCCGATTCAG-3′) for the upstream region of *nosA* and NosA3-SfiI (5′-AATAGGCCTGAGTGGCCCGTATGCTTCGCTTCTTAATG-3′) and NosA3 (5′-CCCACATAGATGTGAGGTTG-3′) for the downstream region and cloned into pCR2.1-TOPO, to generate pKV45 and pKV43 respectively. In a three-fragment-ligation the *argB*-gene from plasmid pSK70 was ligated between the two *nosA*-flanking regions, resulting in vector pKV46. The deletion cassette was amplified with the primers NosA-nested-for (5′-TCGGTACATCTAGGACAGCCAGA-3′) and NosA-nested-rev (5′-CCAGCCGTATCACTTGTCCAT CTAT-3′) and the resulting PCR-product transformed into the arginin-auxotrophic *A. nidulans* strain SRF200. Among 60 transformants, analysed by PCR, one displayed homologous integration of the deletion cassette at the *nosA* locus. As primers for the indicative PCR we used oligonucleotides derived from the *argB* gene: arg5 (5′-TGAGAAATGAT

Table 2. Plasmids used in this study.

Plasmids	Construction	Source
pCR2.1	Cloning vector	Invitrogen (NV Leek, the Netherlands)
pENTR/D-TOPO	GATEway TOPO cloning vector	Invitrogen (NV Leek, the Netherlands)
pHHgfp4	2.5 kb of *hxtA* promoter fusion with *sgfp*	Wei *et al.* (2004)
pJH19	*gpd(p)::dsRedT4-stuA-NLS, argB;* for *in vivo* staining of nuclei	Toews *et al.* (2004)
pKV43	1 kb 3′-flanking region of *nosA* with SfiI site in pCR2.1	This study
pKV45	1 kb 5′-flanking region of *nosA* with SfiI site in pCR2.1	This study
pKV46	*nosA*-deletion construct: flanking regions from pKV43 and pKV45 ligated with *argB* from pSK70	This study
pKV51	*nosA*-ORF without stop-codon in pENTR/D-TOPO	This study
pKV52	*alcA(p)*::*nosA-sgfp*, *argB*; *nosA* from pKV51 via GATEway in pMT-sGFP	This study
pMS19	*gpd(p)::nsdD, pyr4*	This study
pMT-sGFP	GATEway Vector, *alcA(p)::cccB*-box (incl. *attR* sites)::*sgfp*	Toews *et al.* (2004)
pRG1	*N. crassa pyr-4* selectable marker plasmid	Waring *et al.* (1989)
pSK70	*argB* with SfiI sites	This study

TCGTGAATG-3′) and arg3 (5′-GACTCTCCTCATTCCATAC-3′) and the *nosA* internal primers int1 (5′-CCTGAGTT CGAATATGGC-3′) and int2 (5′-CAGGGCTTGGCATAGTTG-3′) (Fig. 3). The Δ*nosA* strain (SKV31) was crossed and the progeny strains analysed by Southern blot. In all strains arginin-prototrophy was linked to the *nosA* deletion.

Tagging of NosA with GFP

The complete *nosA*-ORF was amplified with primers NosA-GW-for (CACCATGCCGGCAGCACCGAGA, underlined are the four bases needed for site-directed TOPO-cloning) and NosA-GW-rev (AAGAAGAAGGTAGTTCCAACC) with the *proof reading* Phusion-Polymerase (Finnzymes, Oy, Espoo, Finnland) and cloned into vector pENTR/TOPO (Invitrogen, Karlsruhe) resulting in pKV51. Sequencing of the *nosA* insert was done commercially by MWG Biotech (Ebersberg). Fusion of NosA with sGFP at the C-terminus was done with the GATEway cloning system and vector pMT-sGFP (Toews *et al*., 2004) resulting in vector pKV52. This plasmid was transformed into the *A. nidulans* strain SRF200.

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.

Light microscopy

For live-cell imaging, cells were grown in glass-bottom dishes (World Precision Instruments, Berlin, Germany) in 2 ml of medium, either MM + 2% glycerol + pyridoxine and/or arginine or MM + 2% ethanol (or threonine) + pyridoxine and/or arginine. Cells were incubated at 30°C for 15 h and 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 analysed with a Hamamatsu Orca ER II camera system and the Wasabi software (version 1.2).

Acknowledgements

We thank Dr K.-H. Rexer (Philipps-University of Marburg) for the assistance for taking SEM pictures. We thank Dr K.S. Chae, Chonbuk National University, Chonju, Chonbuk, Korea for sending us the *A. nidulans nsdD*-deletion strain. We thank Dr P. Ricke (MPI Marburg) for help with the phylogenetic trees. This work was supported by the SFB 395, the Deutsche Forschungsgemeinschaft (DFG), the Max-Planck-Institute for terrestrial Microbiology and the special programme ‘Lebensmittel und Gesundheit’ from the ministry of Baden-Württemberg.

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