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The $Zn(II)_2Cys_6$ Putative *Aspergillus nidulans* Transcription Factor Repressor of Sexual Development Inhibits Sexual Development Under Low-Carbon Conditions and in Submersed Culture

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ABSTRACT

Here we have characterized the putative $Zn(II)_2Cys_6$ transcription factor RosA from the filamentous fungus *Aspergillus nidulans*. The *rosA* gene encodes a protein of 713 aa, which shares 38% sequence similarity to Pro1 from *Sordaria macrospora*. In contrast to Pro1, which promotes the transition from protoperithecia to perithecia, RosA is a negative regulator of sexual development in *A. nidulans*. Transcript levels of *rosA* were usually very low and were only transiently upregulated upon carbon starvation and at 12 hr of asexual development. Deletion of *rosA* only slightly induced fruiting-body formation under standard culture conditions, but enabled sexual development under low-glucose and high-osmolarity conditions and the production of Hülle cells under submersed growth conditions. Stimulation of fruiting-body formation on agar surfaces was dependent on *veA*. In Δ*rosA* strains, transcript levels of the sexual developmental regulators *nsdD*, *veA*, and *stuA* were increased. Overexpression of *rosA* led to a reduction of hyphal growth and to a *fluffy* phenotype. Post-transcriptional regulation of RosA, with a regulated accumulation in the nucleus, was shown using a RosA-GFP fusion protein. We propose that RosA represses sexual development upon integration of several environmental signals.

SPORE formation is a common strategy of many fungi to cope with unfavorable conditions or to propagate in the environment and conquer new habitats. The mechanisms of fungal spore formation can be quite different among genera but usually involve the generation of specialized morphological structures. A model fungus to study spore formation at the molecular level is the euascomycete *Aspergillus nidulans* (Adams *et al.* 1998; Fischer 2002). This mold grows vegetatively through hyphal extension and produces spore-bearing structures, called conidiophores, after a defined period of time. This asexual developmental program occurs at a water-air interface and requires the presence of red light. In addition to the asexual developmental program, *A. nidulans* is able to undergo sexual reproduction with or without prior mating with another strain (Braus *et al.* 2002). The first visible sign of sexual differentiation is the formation of a nest-like structure made of so-called Hülle cells. Underneath these thick-walled cells, primordia develop and mature to form a ripe cleistothecium, also known as the fruiting body. Within the cleistothecia nuclear fusion and subsequent meiosis and ascospore formation occurs (Sohn and Yoon 2002).

Abiotic and biotic factors, such as a water-air interface, light, CO_2 concentration, nutritional status, and a fatty-acid-based and probably a peptide-based pheromone system, are responsible for the developmental decisions determining sexual or asexual reproduction (Zonneveld 1977; Eckert *et al.* 1999; Braus *et al.* 2002; Calvo *et al.* 2002; Dyer *et al.* 2003; Lara-Ortiz *et al.* 2003; Tsitsigiannis *et al.* 2004). Recently, it was discovered that the concentration of reactive oxygen species (ROS) could be a critical factor (Scherer *et al.* 2002). A NADPH oxidase (NoxA), which generates ROS in *A. nidulans*, was reported to be necessary for sexual development (Lara-Ortiz *et al.* 2003).

All factors are integrated and transduced into physiological and morphological responses via G-protein-coupled receptor systems, a MAP kinase and cAMP-signaling cascades, and a number of putative transcription factors (Han and Prade 2002; Kawasaki *et al.* 2002; Wei *et al.* 2003; Han *et al.* 2004). Some of these signaling components were discovered through mutant analyses. Thus, *stuA* mutants display a strong defect in asexual sporulation, have shorter conidiophores, and fail to produce metulae and spore-producing phialides (Miller *et al.* 1992). Instead, spores are directly produced from the vesicle. The StuA protein is a helix-loop-helix transcription factor, which appears to repress the conidiophore-specific regulators *abaA* (Dutton *et al.* 1997) and *brlA* (Busby *et al.* 1996). In addition to the asexual phenotype, Δ*stuA* strains fail to produce sexual reproduc-

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TABLE 1

***A. nidulans* and *E. coli* strains used in this study**

Strain	Genotype	Source
FGSC26	*biA1; veA1*	Fungal Genetics Stock Center (Kansas City)
WIM126	*pabaA1, yA2; veA*$^+$	Champe and Simon (1992)
SRF200	*pyrG89;* Δ*argB::trpC*Δ*B; pyroA4; veA1*	Karos and Fischer (1999)
RMS011	*pabaA1, yA2;* Δ*argB::trpC*Δ*B; veA1*	Stringer *et al.* (1991)
SKV6	RMS011 transformed with pKV12; *yA2, pabaA1; veA1; alcA(p)::rosA*	This study
SKV8	SRF200 transformed with pKV18; *pyrG89; pyroA4, veA1;* Δ*rosA::argB*	This study
SKV12	Cross between SKV8 × RMS011, *veA1;* Δ*rosA::argB*	This study
SKV29	Cross between WIM126 and SRF200; strain selected with no marker, only *veA1*	This study
SKV30	SKV8 cotransformed with Mut(p)-GFP and pRG1 *pyroA4, veA1;* Δ*rosA::argB; mutA(p)::sGFP*	This study
SKV103	Cross between SRF200 × WIM126, progeny strain *pyrG89; pyroA4, veA*$^+$	This study
SKVw3	Cross between SKV8 × WIM126; Δ*rosA::argB*; *veA*$^+$	This study
SKV48	SRF200 transformed with pKV72; Δ*argB::trpC*Δ*B; pyroA4, veA1; alcA(p)::GFP::stuA(NLS)-rosA*$^{294\text{-}449}$	This study
SKV47	SRF200 transformed with pKV71; Δ*argB::trpC*Δ*B; pyroA4, veA1; alcA(p)::GFP::stuA(NLS)*	This study

tion structures and are therefore self-sterile. In this line, StuA has been found to be an activator of a catalase-peroxidase gene, *cpeA*, expressed in Hülle cells (Scherer *et al.* 2002). Another regulator discovered after mutant analysis is the protein NsdD, a putative GATA-type transcription factor (Chae *et al.* 1995; Han *et al.* 2001). The *nsdD* deletion strains are unable to form cleistothecia, while their overexpression causes an increase of the number of fruiting bodies on solid medium as well as masses of Hülle cells in liquid culture. A gene with a key role in the regulation of *A. nidulans* development is *veA*, which mediates the light response (Käfer 1965; Mooney and Yager 1990). *A. nidulans* normally conidiates in the presence of red light while it undergoes sexual reproduction under permanent dark conditions. When *veA* is mutated, conidiophore development is enhanced and occurs even in the dark. Concomitantly, sexual reproduction is lowered. Likewise, *veA* overexpression causes the induction of the sexual cycle even under inappropriate conditions, such as high osmolarity or in liquid medium. This suggests a complex function for this regulator in both sexual and asexual development (Kim *et al.* 2002). A putative transcription factor that plays a role in both reproduction strategies is *dopA*. Corresponding mutants grow with abnormal vegetative hyphae and produce aberrant conidiophores. Conidiation is drastically reduced due to delayed and asynchronous initiation of asexual development. Sexual reproduction in this mutant is completely abolished (Pascon and Miller 2000). The homeodomain C_2/H_2-Zn^{+2} finger transcription factor SteA, with similarity to the *Saccharomyces cerevisiae* Ste12 protein, is also an important regulator of sexual reproduction in *A. nidulans* (Vallim *et al.* 2000). Its deletion causes a developmental block of fruiting-body formation at the Hülle cell stage. Ascogenous hyphae or cleistothecia are not produced.

Recently, a role for the signalosome in sexual development was discovered (Busch *et al.* 2003). The COP9 signalosome is a conserved multiprotein complex involved in the regulation of development in mammals and plants. Deletion of two of the COP9-signalosome components, *csnD* and *csnE*, in *A. nidulans* showed the same specific block in an early stage of cleistothecial formation. However, this signaling module appears to be involved in several other morphogenetic pathways. Despite the accumulated knowledge regarding single signaling components regulating the developmental decision between sexual and asexual reproduction, the links among them are still missing.

Similarly, little is known about regulators involved after the onset of the sexual developmental program. A possible regulator of the process of cleistothecia maturation could be a homolog of the $Zn(II)_2Cys_6$ transcription factor, Pro1, from the homothallic fungus *Sordaria macrospora* (Masloff *et al.* 1999). Mutation of this regulator results in a specific block of perithecium development. We screened the genomic DNA database of *A. nidulans* and identified a gene, *rosA*, whose translation product shared sequence similarity to Pro1. We have characterized the gene and its protein and found, interestingly, that Pro1 and RosA serve different functions in the two ascomycetous fungi. We present evidence that RosA acts as a repressor of sexual development initiation under low-carbon conditions and in submersed culture.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions: Supplemented minimal and complete media for *A. nidulans* were prepared as

TABLE 2

Plasmids used in this study

Plasmids	Construction	Source
pBluescript KS	Cloning vector	Invitrogen (NV Leek, The Netherlands)
pCMB17apx	*alcA*(p)::*eGFP, pyr4*; for N-terminal fusion of GFP to genes of interest	V. Efimov (Piscataway, NJ)
pCR2.1	Cloning vector	Invitrogen (NV Leek, The Netherlands)
pJH19	*gpd(p)::dsRedT4-stuA-NLS, argB;* for *in vivo* staining of nuclei	Toews *et al.* (2004)
pHW	*argB* from pDC1 in pBluescript KS$^-$	H. Wei (Marburg, Germany)
pNRSte1	*pyr4* amplified from pRG1 and cloned into pCR2.1	N. Requena (Marburg, Germany)
pDC1	*A. nidulans argB* selectable marker plasmid	Aramayo *et al.* (1989)
pRG1	*N. crassa pyr-4* selectable marker plasmid	Waring *et al.* (1989)
pMS51	PCR (Expand)-amplified *rosA* with *Xho*I sites in pCR2.1	M. Scherer (Marburg, Germany)
Mut(p)-GFP	Mutanase promoter fused to sGFP	Wei *et al.* (2001)
42D6	Cosmid containing *rosA* (PUI gene bank)	This study
pKV8	*alcA(p)* in *Bam*HI, *argB* in *Not*I in pBluescript KS	This study
pKV12	*rosA* from pMS51 in *Xho*I in pKV8, overexpression construct	This study
pKV15	*Kpn*I/*Xba*I-subclone of *rosA* from cosmid 42D6	This study
pKV18	*argB* from pHW in *Bam*HI/*Eco*RI in pKV15, *rosA* KO-construct	This study
pKV48	~1-kb *rosA* fragment (aa 1–294) in pCMB17apx in *Asc*I/*Pac*I	This study
pKV50	*rosA* full-length ORF (aa 1–713) in pCMB17apx *in Asc*I/*Pac*I	This study
pKV57	*rosA* truncated ORF (aa 1–642) in pCMB17apx *in Asc*I/*Pac*I	This study
pKV58	*rosA* truncated ORF (aa 1–449) in pCMB17apx in *Asc*I/*Pac*I (pKV50 *Bgl*II cut, religated)	This study
pKV60	*rosA* DNA-binding site + NLS (aa 1–80) in pCMB17apx in *Asc*I/*Pac*I	This study
pMut-p-gfp	*mutA* promoter fused with *sGFP* in pBluescript KS−	Wei *et al.* (2001)
pYU8	Dominant-active *fadA*-allele	Yu *et al.* (1996)
pKV72	*eGFP::stuA(NLS)-rosA*$^{294-449}$ in pCMB17apx	This study
pKV71	*eGFP::stuA(NLS)* in pCMB17apx	This study

described, and standard strain construction procedures were used (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 and cosmids 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, ID) 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 according to the manufacturer's protocols (GibcoBRL Life Technologies, Paisley, Scotland, UK). DNA and RNA analyses (Southern and Northern hybridizations) were performed as described (Sambrook and Russel 1999).

Deletion of *rosA*: The *rosA* open reading frame was replaced with the *argB* marker. The *argB* auxotrophic marker was cloned into *Bam*HI-*Eco*RI sites of pKV15. This results in deletion of a 2.5-kb restriction fragment including the *rosA* open reading frame with only 100 bp upstream of the stop codon left in the subclone flanked by 1.2- and 2.5-kb border regions, respectively. The deletion construct (pKV18) was linearized with *Kpn*I/*Xba*I and transformed into the arginine-auxotrophic *A. nidulans* strain SRF200. To verify the disruption event, we used the following three primers: primer 1, 5′-CCCAGCTCGAC CATGCCG-3′; primer 2, 5′-GCCCTCGAGAAATATTAAAGC AGAAGATAGTGCC-3′; and primer 3, 5′-CTGAGAAATGATT CGTGAATG-3′.

GFP labeling of RosA: For the N-terminal fusion construct, the *rosA* open reading frame (starting from ATG and ending ~750 bp after the stop codon) was amplified from genomic DNA as template and Pfu ultra polymerase (Stratagene, La Jolla, CA). Primers used were RosA-for-*Asc*I (5′-GGCGCGCCCGGG ATGTCAGCTCTCGCCGGAC-3′) with an *Asc*I site added at the 5′-end and RosA-long-rev-*Pac*I (5′-TTAATTAAGAATGTTTCA GACTCATGTAG-3′), introducing a *Pac*I site at the 3′-end. The PCR product was incubated for 30 min at 72° with Taq-Polymerase to add a T at the 5′-end and afterward cloned into the TOPO-TA cloning vector pCR2.1 (Invitrogen, Karlsruhe, Germany). The clone was sequenced by MWG Biotech and the *rosA* was cloned in the pCMB17apx plasmid (kindly provided by V. Efimov, Piscataway, NJ) into *Asc*I-*Pac*I sites behind the *alcA* promoter and the *GFP* gene (pKV50). Truncated versions of GFP-RosA were obtained by digests with *Pst*I (642 aa, pKV57) and *Bgl*II (449 aa, pKV58) and by religation. This results in an additional 18 aa for pKV58 and 33 aa for pKV57 due to translation of the polylinker of the plasmids. For the two short constructs (80 aa, pKV60 and 294 aa, pKV48), parts of the *rosA* open reading frame were amplified with genomic DNA as template, the same forward primer as above, RosA-for-*Asc*I, and the reverse primers RosA-80-aa-rev (5′-TTAATTAA CTATCGGTATTCGCAGCTGAC-3′) or RosA-300-aa-rev (5′-TTA ATTAATTAGTCGACAAAGTGATAGAGTAG-3′), both containing an additional *Pac*I site and a stop codon. Cloning of the PCR products was the same as already described for pKV50.

All GFP constructs were transformed into the two *pyrG* mutant strains SRF200 (*veA1*) and SKV103 (*veA*$^+$).

Overexpression of *rosA*: The *rosA* open reading frame (from 70 bp upstream of the ATG to the predicted stop codon) was amplified with Expand polymerase (Roche, Mannheim) and the primers RosA-5′ (5′-TACCTCGAGCACCTGTCGCAGA TATCTGG-3′) and RosA-3′ (5′-GCCCTCGAGAAATATTAA AGCAGAAGATAGTGCC-3′), both containing an additional *Xho*I site. The PCR product was first cloned into the TOPO-TA cloning vector pCR2.1 (Invitrogen) and from there into the *Xho*I site of the overexpression vector pKV8, containing the inducible *A. nidulans alcA* promoter and the auxotrophy marker *argB*. The *rosA* overexpression construct (pKV12) was transformed into the arginine-auxotrophic strain RMS011, and transformants were grown under repressing (glucose) and inducing (ethanol) conditions.

Light microscopy, live-cell image acquisition, and analysis: Cells were grown in glass-bottom dishes (World Precision Instruments, Berlin) in 2 ml of minimal medium (MM) without glucose + 2% glycerol + pyridoxine and/or arginine or MM + 2% ethanol (or threonine) + pyridoxine and/or arginine medium. Cells were incubated at 37° for 10 hr and images were captured at RT with an Axiophot microscope (Zeiss, Jena), a Planapochromatic ×63 oil immersion objective lens, and a 50 W Hg lamp. Images were collected and analyzed with a Hamamatsu Orca ER II camera system, and the Wasabi software (version 1.2).

Electron microscopy: For scanning electron microscopy (SEM), colonies grown on ethanol 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.

RESULTS

Molecular cloning of the *rosA* gene: We used the protein sequence of *S. macrospora* Pro1 to screen the *A. nidulans* DNA sequence database at the Whitehead Institute (Cambridge, MA). We found two sequences with similarity to Pro1 and used them to isolate a corresponding cosmid (PUI library kindly provided by B. Miller, University of Idaho, Moscow, ID). The characterization of one of these genes, *rosA*, is presented here, while the results obtained for the second gene will be published elsewhere. After subcloning of a 6-kb *Xba*I/*Kpn*I restriction fragment, the full *rosA* genomic locus was sequenced on both strands, which confirmed the sequence obtained in the genome project. RT-PCR was used to generate a corresponding cDNA, and two introns of 82 and 45 bp were detected at the 5′-end of the gene. The border sequences of the introns were in agreement with the consensus sequences described for fungi (Gurr *et al.* 1987). The position of the larger intron is conserved in orthologs of *S. macrospora*, *Sordaria brevicollis*, and *Neurospora crassa* (Figure 1). The gene from *A. nidulans* was named *r*epressor *o*f *s*exual development (*rosA*) because of the phenotype of the corresponding deletion strain (see below).

The deduced RosA protein has a molecular mass of 79.6 kD and a calculated isoelectric point of 7.6. At the N terminus of the protein, we detected the highly conserved $Zn(II)_2Cys_6$ motif and a putative bipartite nuclear localization signal, which overlaps with the predicted DNA-binding domain (Figure 1). In addition, we found a threonine-rich region (aa 178–236) and two putative PEST sequences (PESTfind analysis program at http://www.expasy.org; amino acids 133–253 and 646–659). The identity of the amino acid sequence of the $Zn(II)_2Cys_6$ region to the same motif in other transcription factors was between 55 and 70%. In the case of Pro1 from *S. macrospora* and orthologs from other fungi, long stretches of identical amino acid sequences were found in the C terminus of the proteins in addition to the identity of the DNA-binding motifs (Figure 1). The region from amino acid 80 to 352 displayed weak similarity (20% identity) to the corresponding regions of *S. macrospora* or *N. crassa*. The protein sequence identity between full-length RosA and Pro1 of *S. macrospora* or of the Pro1 orthologs from *N. crassa* and *S. brevicollis* was ~38%. In contrast, sequence identities among the latter three Pro1 proteins are ~90%, and the corresponding genes complement the *pro1* mutation in *S. macrospora*. In addition to Pro1, Masloff *et al.* (2002) detected another similar protein, Pro1A, in the database of *N. crassa*. This transcription factor displayed only 40% identical amino acids to Pro1 and could not complement the *pro1* mutant phenotype of *S. macrospora*. The identity between *A. nidulans* RosA and Pro1A was only 21%. In two other Aspergilli, *A. fumigatus* and *A. oryzae*, we found proteins with identities to RosA of 42.5 and 36.0%, respectively. We found no significant hits in the genomes of *S. cerevisiae*, *Schizosaccharomyces pombe*, the filamentous ascomycete *Ashbya gossypii*, or the basidiomycetous fungi *Ustilago maydis* and *Coprinus cinereus*.

Deletion of *rosA*: To assess the role of RosA, we deleted the gene from the genome. In the 6-kb *Kpn*I/*Xba*I restriction fragment, a 2.5-kb *Bam*HI/*Eco*RI of the *rosA* open reading frame was replaced by the nutritional marker gene *argB*. With this cloning strategy, we removed a DNA fragment containing 400 bp upstream of the ATG and the open reading frame, except the last 100 bp to the stop codon. The construct (pKV18) was introduced into the *argB* auxotrophic strain (SRF200) and transformants were screened by Southern blot for a homologous recombination event. We found one colony, among 80 transformants, with the expected banding pattern (Figure 2). The gene replacement event was confirmed by PCR and other restriction digests. To make sure that the strain did not contain any other mutations, we crossed it to an *argB* auxotrophic strain (RMS011) and selected strains from the progeny with the *rosA*-deletion event. Phenotypic inspection of independent Δ*rosA* progeny strains revealed no significant differences to the wild-type strain regarding timing of asexual or sexual development, morphology of the conidiophores, number of conidia, or number of cleistothecia or ascospores, when grown under standard laboratory

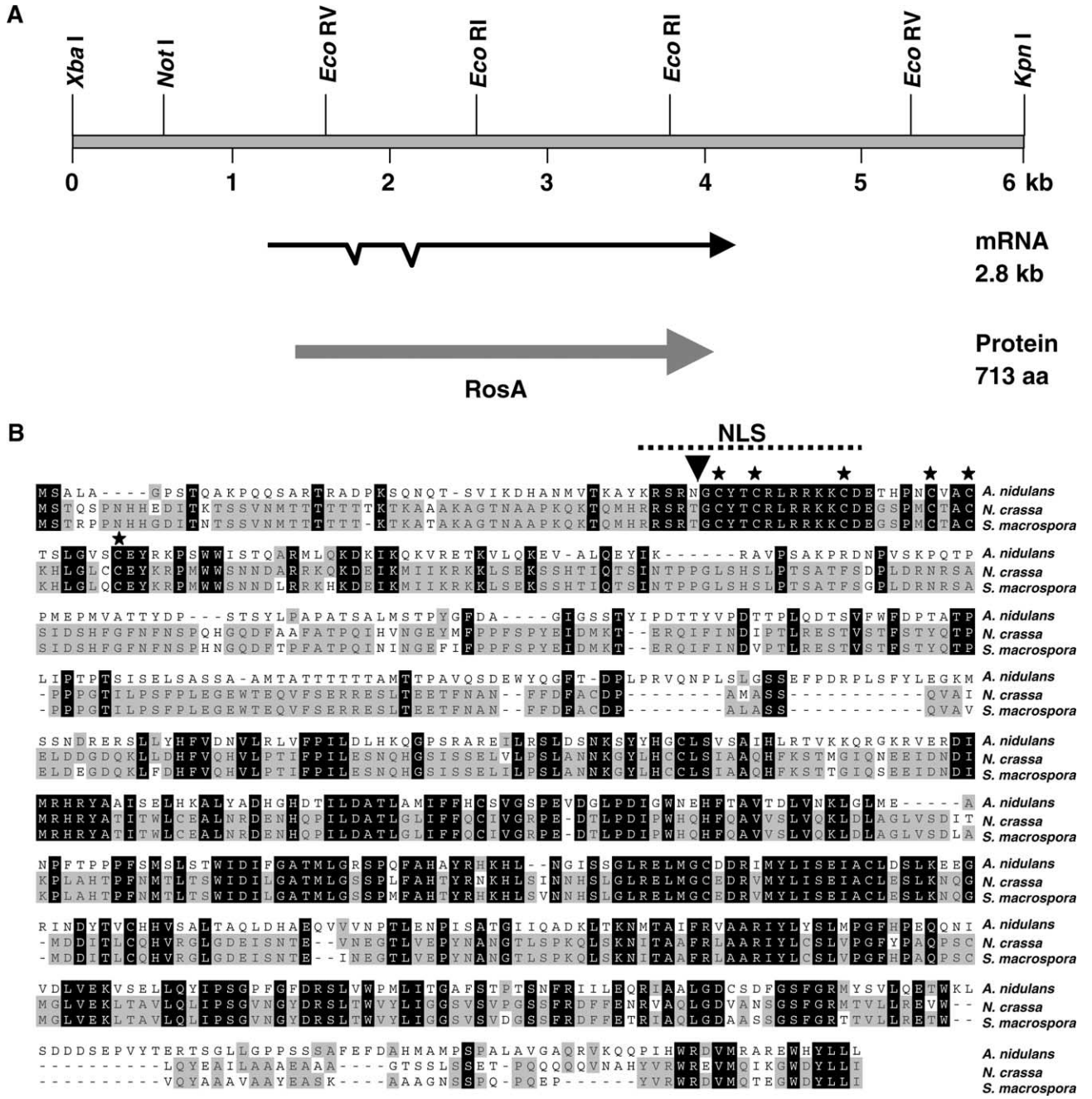

FIGURE 1.—(A) Scheme of the *rosA* gene locus. (B) Alignment of RosA with Pro1 from *S. macrospora* (accession no. Q9UVG3) and the homolog from *N. crassa* (accession no. Q9P326). Shaded background indicates amino acids that were identical in two sequences and solid background indicates amino acids that matched in all three proteins. 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 the conserved intron is indicated by an arrowhead. The predicted bipartite NLS is indicated by a dashed line above the sequence. The *A. nidulans* sequence is available under accession no. CAD58393.

conditions on agar plates. Because common laboratory strains, such as SRF200 or RMS011, harbor a mutation in the developmental regulator *veA*, we wanted to prove whether the *rosA* deletion causes a phenotype in a *veA*$^+$ background. We crossed SKV8 with WIM126 and selected a Δ*rosA*, *veA*$^+$ strain (SKVw3) with no other mutations and compared it to the *rosA*$^+$, *veA*$^+$ strain FGSC4. The presence of the *veA*$^+$ allele was confirmed by sequence analysis of the corresponding part of the gene. We incubated the strains in the presence and absence of light and found that the number of cleistothecia was increased in the *rosA*-deletion strain in the dark. This result suggests that *rosA* partially represses sexual development in *A. nidulans* and that this function depends

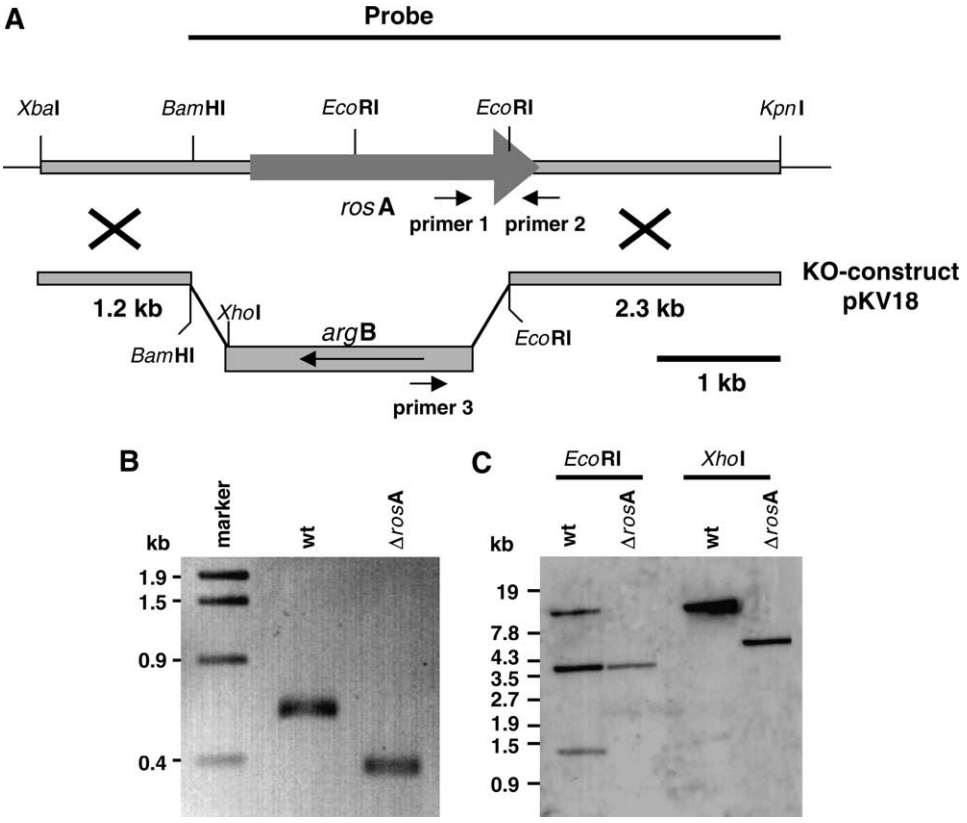

FIGURE 2.—Deletion of the *rosA* gene. (A) Scheme of the knockout construct pKV18. (B) PCR analysis for the demonstration of the *rosA*-deletion event using the three oligonucleotides indicated in A. PCR fragments were separated on a 1% agarose gel and stained with ethidium bromide. (C) Southern blot analysis of a *rosA*-deletion strain. Genomic DNA of a wild-type (SRF200) and a *rosA*-deletion strain was isolated, restricted with *Eco*RI (left blot) and *Xho*I (right blot), separated on a 1% agarose gel, blotted, and hybridized with the ^{32}P-labeled probe indicated in A.

on *veA*. The phenotypic difference between wild type and Δ*rosA* strains became more drastic when we changed the concentration of the carbon source or grew them in the presence of 0.6 M KCl. When the glucose concentration was reduced from 100 to 10 mM, cleistothecia formation was dramatically reduced in wild type, whereas in *rosA*-deletion strains mature cleistothecia with fertile ascospores were still produced (Figure 3). Interestingly, cleistothecia were located in the border region of the Δ*rosA* colony rather than in the center as in wild type. We quantified this effect by counting the Hülle cell nests in a circle of 0.5 cm diameter at a distance of 0.5 cm from the colony border and found 26 (±12) for Δ*rosA* and 0.7 (±2) for wild type. Similarly,

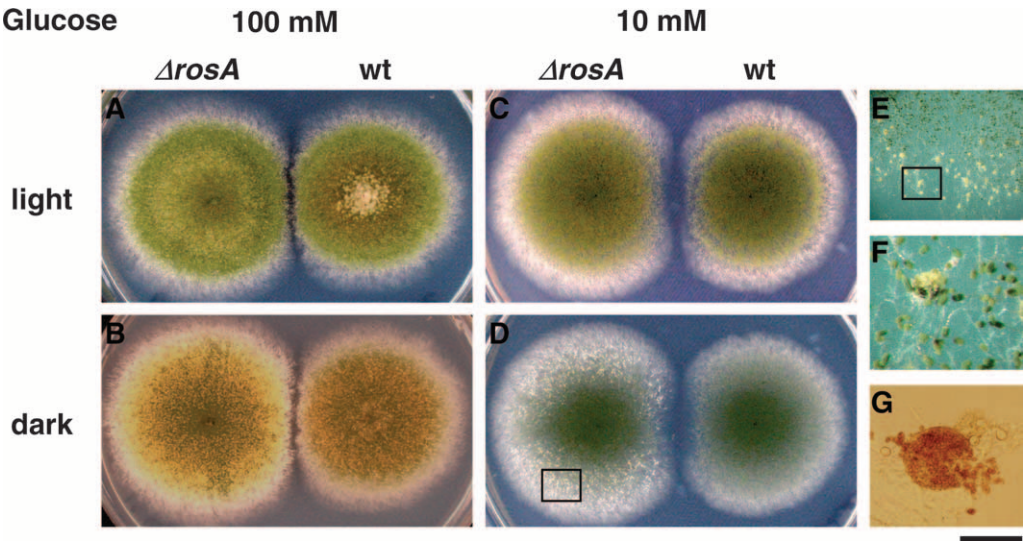

FIGURE 3.—Phenotype of a *rosA*-deletion strain. Point-inoculated Δ*rosA* strain pKVw3 and wild-type FGSC4, both *veA*$^{+}$, were grown for 4 days at 37° on solid minimal medium. (A) No difference between Δ*rosA* and wild type is observed on agar plates containing 100 mM glucose and incubated in the light. (B) On agar plates incubated in the dark, the number of cleistothecia is increased in the Δ*rosA* strain. (C and D) Comparison of wild-type and Δ*rosA* strains on low-glucose medium (10 mM). Neither wild type nor the Δ*rosA* strain produce cleistothecia in the light. In the dark, the Δ*rosA* strain undergoes sexual development and produces cleistothecia. (E) Enlarged view of the region boxed in D. (F) Further enlargement of the boxed area in E shows a cleistothecium embedded in yellow Hülle cells. (G) Crushed cleistothecium, which released mature asci and red ascospores. Bar, 1.5 cm in A–D; 2 mm in E; 400 μm in F; and 200 μm in G.

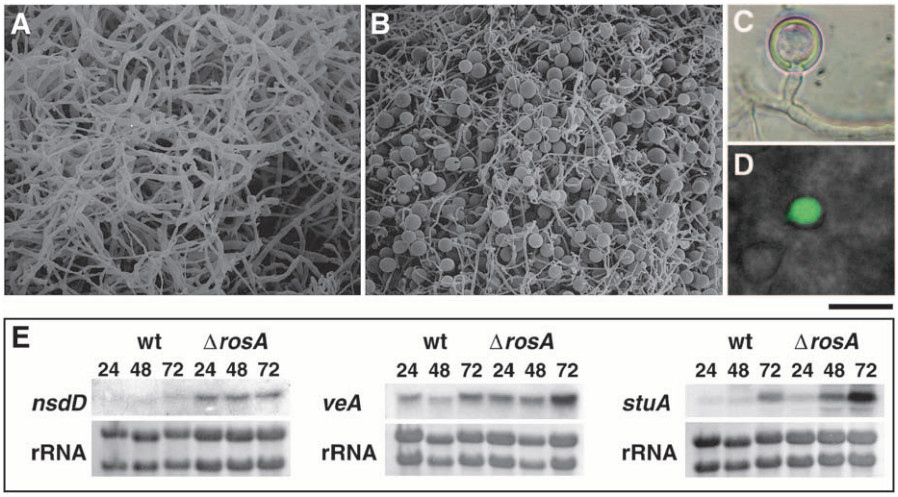

FIGURE 4.—Phenotype of a *rosA*-deletion strain in liquid minimal medium. Medium was inoculated with 10^6 spores/ml and incubated on a horizontal shaker (200 rpm) for 72 hr. (A) Mycelia of wild type (SKV29) and (B) mycelia of the *rosA*-deletion strain SKV12 as seen in the scanning electron microscope. (C) Phase-contrast picture of an individual Hülle cell. (D) A bulk of Hülle cells of the MutA(p)-GFP-expressing strain SKV30 generated in liquid culture. The picture was taken with a combination of phase-contrast and fluorescence illumination. The Hülle cell in the middle shows green fluorescence in the cytoplasm. (E) Northern blot analysis of three developmental regulators during growth in submersed culture. Cultures of wild type (wt) and mutant (Δ*rosA*) were harvested after 24, 48, and 72 hr, RNA isolated, and subjected to Northern blotting. The membranes were hybridized with gene-specific DNA fragments labeled with ^{32}P. Prior to hybridization, rRNA was stained with methylene blue as loading control.

addition of 0.6 M KCl still allowed the production of mature cleistothecia with fertile ascospores in *rosA*-deletion strains, while in wild-type strains cleisthothecia production was much more strongly inhibited.

Since it is known that development is repressed in submersed culture, we asked whether a Δ*rosA* strain would be able to initiate the sexual cycle under these conditions. Indeed, we found Hülle cell formation after 48 hr of cultivation of the Δ*rosA* strain in liquid culture (Figure 4). Although the number of Hülle cells increased after prolonged incubation, further developmental stages, such as cleistothecia were not observed. This phenotype was independent of the *veA* gene and fully complemented with a genomic copy of *rosA* (pKV15; results not shown).

The question of whether the Hülle cells produced in submersed culture were physiologically active was addressed by analysis of the expression of a Hülle-cell-specific gene, the α-1,3-glucanase *mutA* (WEI *et al.* 2001). A corresponding GFP fusion construct (Mut(p)-gfp) was transformed into a *rosA*-deletion strain. We analyzed several independent transformants for GFP fluorescence, because the plasmid integrates ectopically into the genome of *A. nidulans*, and expression of the construct might vary depending on the integration site and the copy number. Hülle cells appeared in liquid medium after 48 hr. About 10% of the cells showed GFP fluorescence. This small number might reflect different physiological states of the Hülle cells, but nevertheless suggests that the cells are viable and active (Figure 4; WEI *et al.* 2001).

Interaction of *rosA* with other developmental regulators: The phenotype of Hülle cell production in submersed culture was already described for strains overexpressing either *nsdD* or *veA* (HAN *et al.* 2001; KIM *et al.* 2002). To test whether deletion of *rosA* causes an induction of a *nsdD* or a *veA* transcript, we performed Northern blot analyses. Mycelium (SKVw3) was grown for 24, 48, and 72 hr in liquid culture and then processed for RNA analysis. In the wild-type strain (FGSC4), *nsdD* was expressed at a low level at all time points while it was upregulated in the Δ*rosA* strain (two independent experiments). Similarly, the *veA* gene was slightly induced in the absence of *rosA*. StuA, which induces Hülle-cell-specific genes (SCHERER *et al.* 2002), was also upregulated in the Δ*rosA* strain in comparison to wild type after 48 and 72 hr (Figure 4).

Overexpression of *rosA*: To overexpress *rosA*, we replaced the endogenous promoter by the highly inducible *alcohol dehydrogenase* (*alcA*) promoter from *A. nidulans*. We introduced the resulting construct (pKV12) as a single copy into *A. nidulans* wild type (RMS011). Under repressing conditions (growth on 2% glucose) no significant difference with respect to hyphal growth or asexual or sexual development was observed. However, induction of the gene with 2% ethanol (or threonine) as carbon source resulted in a drastic developmental phenotype, which was also observed when the induction level was decreased by using 2% glycerol instead of ethanol (results not shown). Transferred colonies grew only with a reduced extension rate in comparison to the wild type and further development was completely inhibited. Instead, massive production of aerial hyphae occurred and led to a cotton-like appearance of the colonies (Figure 5). Of 20 transformed strains, 16 showed this phenotype, suggesting that the phenotype was not due to disruption of a developmental gene upon ectopic integration of the overexpression construct. A similar phenotype is well known for a class of mutants called *fluffy*. This class is composed of, *e.g.*, transcription factors and other genes encoding proteins involved in signal transduction. One candidate of the latter group is *fadA*, which codes for the α-subunit of a heterotrimeric G-protein (YU *et al.*

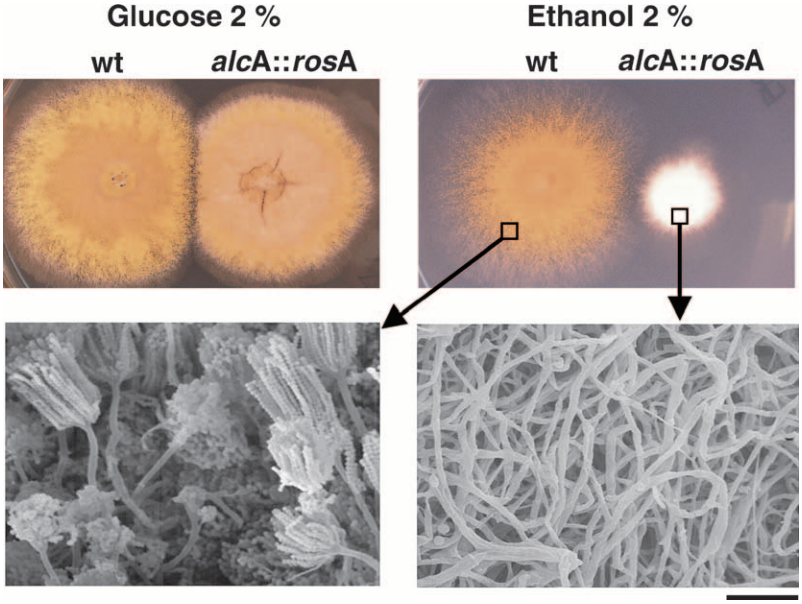

FIGURE 5.—Overexpression of the *rosA* gene. Comparison of a wild type (RMS011) and SKV6 (*alcA(p)::rosA*) grown on glucose (left) and ethanol medium (right). Plates were incubated for 4 days at 37°. (Bottom, left and right) Scanning electron microscopic pictures of a wild type (left) and the overexpression strain (right). Bar represents 1.5 cm (colonies) and 100 μm (bottom, left and right).

1996). When a constitutively active allele of *fadA* is introduced into an *A. nidulans* wild-type strain, the colony adopts a fluffy appearance. To analyze whether RosA acts as a transcription factor downstream of the activated G-protein, we introduced a constitutively active allele of *fadA* into a *rosA*-deletion strain (SKV8) by cotransformation of pYU8 with the *pyr4*-containing plasmid pRG1. Among the obtained transformants ~40% of the clones displayed the fluffy phenotype. The same percentage of fluffy colonies was obtained after cotransformation of pYU8 into the wild-type strain RMS011. This suggests that FadA signaling is independent of RosA.

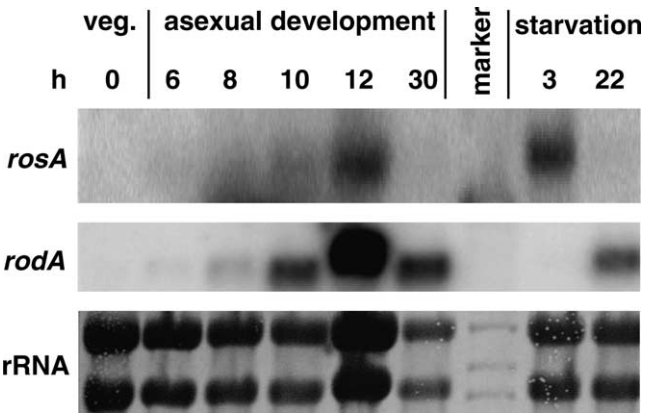

FIGURE 6.—Transient expression of *rosA* during development and upon starvation. *A. nidulans* was induced for differentiation and mycelium harvested at the time points indicated. In addition, mycelia grown in liquid culture were harvested after 20 hr, washed, and transferred in medium lacking any carbon source. After 3 hr and after 22 hr of incubation at 37° mycelium was harvested and processed for transcript analysis. RNA was isolated from all samples and 15 μg processed for Northern blots using a 1-kb ^{32}P-labeled *rosA*-specific probe of the open reading frame generated by PCR. As a control we have hybridized the RNA of the asexual stage and after starvation with the *rodA*-specific probe generated by PCR. As a further loading control we stained the ribosomal RNA after transfer to the membrane with methylene blue.

Transcription of *rosA* is upregulated during asexual development and upon carbon starvation: The regulation of *rosA* expression was studied by Northern blot and RT-PCR analyses of mycelium harvested at different developmental stages. To synchronize development, hyphae were grown in liquid culture for 20 hr and then filtered through a miracloth membrane and transferred to the surface of an agar plate. Under those conditions, asexual development is initiated immediately after transfer and stalks of conidiophores are visible after 6 hr; metulae, phialides, and some spores after 12 hr; and after 20 hr asexual development is completed. Sexual development is then initiated after 48 hr. The *rosA* transcript was detectable only in the hyphal stage using nested PCR with cDNA as template, which was generated in a first reaction by RT-PCR (data not shown). The *rosA* transcript became detectable by Northern blot analysis only in early developmental stages and peaked in the 12-hr sample. These results suggest that the expression is tightly developmentally regulated (Figure 6). Since the carbon supply in developing cultures may be limiting, we tested whether *rosA* expression is affected by the nutritional status of the mycelium. We grew *A. nidulans* for 20 hr in liquid culture, filtered the mycelium, washed it with medium without glucose, and incubated the material for 3 hr in liquid medium lacking any carbon source. The *rosA* gene was transiently induced after 3 hr of carbon starvation (Figure 6). As a control for the *rosA* expression pattern, we probed the same Northern blot with the *rodA* gene. This gene encodes a hydrophobin, which forms the rodlet layers of the

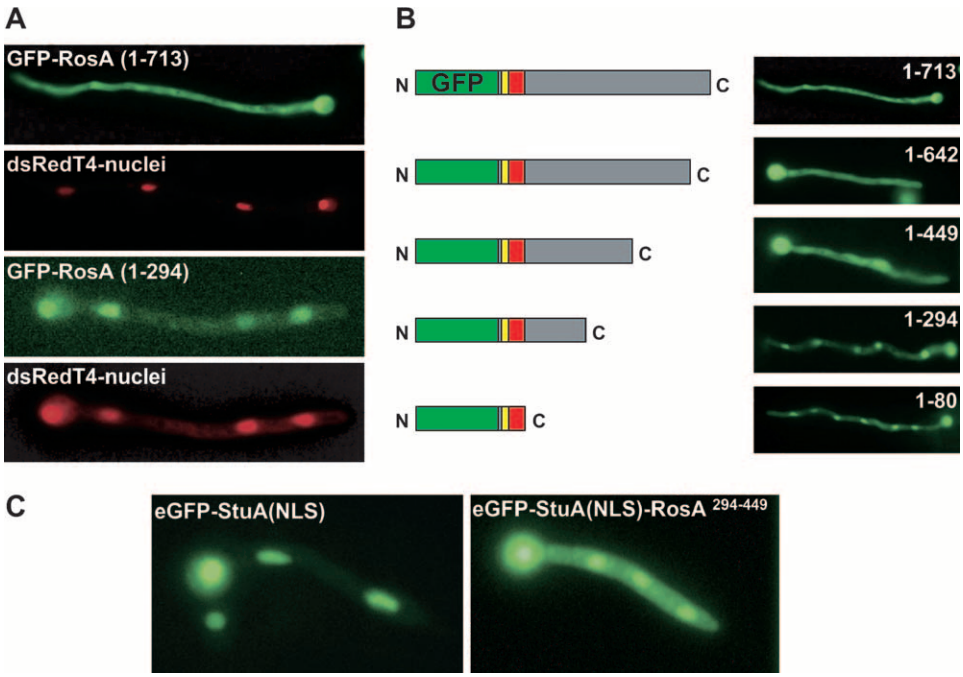

FIGURE 7.—Subcellular localization of RosA as GFP-fusion protein. (A) Expression of full-length RosA and a truncated version as indicated with the amino acid numbers in parentheses. Nuclei were visualized with nuclear-targeted dsRedT4. (B, left) Scheme of different RosA versions fused to GFP at the N terminus. (B, right) Localization of the corresponding constructs shown on the left. The sizes of the proteins are indicated in the pictures. All RosA fusion proteins were expressed under the control of the *alcA* promoter and grown for 10 hr at 37° in medium with 2% ethanol as carbon source. (C) GFP-StuA(NLS) was fused to RosA$^{294\text{-}449}$ and expressed under the control of the *alcA* promoter (pKV72) in strain SRF200, yielding SKV48. As a control GFP-StuA(NLS) (pKV71) used (SKV47). Germlings were grown on ethanol medium as in B.

conidiospores and is thus expressed late during asexual development (STRINGER *et al.* 1991). Surprisingly, the *rodA* transcript was also induced upon prolonged starvation in liquid culture. The *rosA* transcript was not detectable during sexual development either in Northern blot analysis or by nested RT-PCR (data not shown).

RosA localization: The putative transcriptional regulator RosA contains a potential nuclear localization signal (NLS) overlapping with the Zn binuclear cluster DNA-binding domain at the N terminus of the protein (Figure 1). To check whether the NLS is functional, we fused RosA with GFP. Expression under the endogenous *rosA* promoter did not give any GFP signal, which is in agreement with the low expression level observed in the Northern blot analysis. Therefore, we placed *rosA* under the control of the inducible *A. nidulans alcA* promoter and fused *GFP* to the 3′-end. Although transformants were grown in the presence of ethanol to induce the *alcA* promoter, we could not detect any GFP signal. When we changed the GFP molecule from the C to the N terminus of the protein, we obtained a GFP signal (Figure 7). Interestingly, the full-length RosA protein did not accumulate efficiently in nuclei, but nuclei were not excluded. This was observed after induction of the construct with ethanol and also after low expression in the presence of glycerol. Thus it seems that only some of the GFP-RosA proteins were able to enter the nucleus. When we successively truncated the C terminus of the protein, we achieved efficient nuclear translocation of versions shorter than 293 amino acids, suggesting that the region from amino acid 294 to 449 negatively affects nuclear import. Nuclear residence was proven through colocalization with a nuclear-targeted dsRedT4 protein (Figure 7). The RosA localization pattern was identical in a *veA1* mutant and in a *veA* wild type. To test whether the RosA$^{294\text{-}449}$ was sufficient to reduce the efficiency of nuclear import of another nuclear protein, we fused this polypeptide to the C terminus of the transcriptional regulator StuA. We are using a GFP fusion of this C-terminal StuA fragment routinely for nuclear labeling (SUELMANN *et al.* 1997). Interestingly, the GFP-StuA protein was not efficiently translocated into the nuclei when the RosA fragment was added (Figure 7).

DISCUSSION

Six-cysteine binuclear cluster DNA-binding domains are a characteristic class of fungal transcription factors (TODD and ANDRIANOPOULOS 1997). In the complete *S. cerevisiae* genome 56 and in *N. crassa* at least 27 (http://www.broad.mit.edu/annotation/fungi/neurospora/gene_by_hmmer.html) different putative $Zn(II)_2Cys_6$ proteins have been identified (SCHJERLING and HOLMBERG 1996; MASLOFF *et al.* 2002). In comparison, 123 potential $Zn(II)_2Cys_6$ binuclear cluster proteins have been annotated in the *A. nidulans* genome database (http://www.broad.mit.edu/annotation/fungi/aspergillus/genes_by_hmmer.html). The DNA-binding motif was first described in the *S. cerevisiae* Gal4 regulator and has been found since in >80 characterized proteins, mainly from ascomycetes (PAN and COLEMAN 1990). Many of these transcription factors have been described as regulators of different metabolic pathways, ranging from carbon metabolism to the synthesis of secondary metabolites (TODD and ANDRIANOPOULOS 1997). In addition, some $Zn(II)_2Cys_6$ proteins have been shown to regulate

developmental processes in fungi. Thus, in *S. cerevisiae* one factor, Ume6, is involved in meiosis and has activating and repressing properties (Rubin-Bejerano *et al.* 1996). Two transcription factors from this family that regulate fungal morphogenesis have been described in *N. crassa* (*fl*) and in *S. macrospora* (*pro1*). When the *fl* gene in *N. crassa* was mutated, the strain failed to form asexual spores, while mutation of *pro1* in *S. macrospora* blocked the formation of mature perithecia (Bailey and Ebbole 1998; Masloff *et al.* 1999). In both cases the loss-of-function phenotype suggested a gene-activating function. This was confirmed in the case of Pro1 by a functional analysis in *S. cerevisiae*, where a transactivation domain was found in the N-terminal half of the protein (Masloff *et al.* 2002).

Here, we describe RosA, a protein of *A. nidulans* with sequence similarity to the Pro1 transcription factor from *S. macrospora,* but serving a distinctly different role from that of Pro1. Whereas a loss-of-function mutation in *S. macrospora* leads to a block in late sexual development, with the formation of aberrant perithecia, we present evidence that *rosA* acts as a repressor of sexual development under low-carbon conditions and in liquid medium in *A. nidulans*. These observed different functions in Sordaria and Aspergillus contrast with the degree of sequence similarity and conservation of one of the two introns, which argues for a common ancestor of these genes. In agreement with the hypothesis of different functions for Pro1 and RosA is a series of experiments in which we tested whether *pro1* and *rosA* could substitute each other. Overexpression of Sordaria *pro1* in *A. nidulans* did not produce the expected fluffy phenotype, and, similarly, transformation of *rosA* into the *S. macrospora pro1* mutant did not rescue the mutant phenotype (S. Pöggeler, personal communication; data not shown).

The *rosA* mutant, in contrast to the wild-type strain, produced mature cleistothecia under low-glucose (10 mm) and high-salt (0.6 m KCl) conditions and initiated the sexual cycle—reflected by the production of Hülle cells—in submersed culture. Whereas the induction of sexual development in Δ*rosA* on agar plates was dependent on *veA*, the production of Hülle cells in liquid medium was *veA* independent. These phenotypic differences suggest that RosA represses sexual development under certain environmental conditions. In agreement with the role of RosA as a repressor of sexual development is the finding that three transcription factors with known roles in sexual development—*nsdD, veA*, and *stuA*—were transcriptionally induced in liquid culture in the Δ*rosA* strain (Figure 4). This result, together with the strong induction of *rosA* 12 hr after the initiation of asexual development, correlates nicely with the findings from Han *et al.* (2001), who observed downregulation of *nsdD* 10 hr after the induction of asexual development. It remains to be studied whether *nsdD, veA*, and *stuA* are direct targets of RosA.

Interestingly, we observed that *rosA* also undergoes a transient upregulation 3 hr after removal of glucose. Since sexual development occurs after the depletion of the carbon source and therefore depends on reserve materials (Wei *et al.* 2001), it could be that RosA inhibits the onset of sexual development after short-term starvation periods. This starvation-mediated induction of *rosA* could explain the upregulation 12 hr after the onset of asexual development, since conidiophores grow into the air and may be carbon limited. However, we still do not know whether the increase of the *rosA* transcript is restricted to some cell types such as the conidiophores or the substrate mycelium.

The sexual-development-repressing properties of RosA resemble those of the LsdA protein recently characterized (Lee *et al.* 2001). Deletion of *lsdA* led to the induction of sexual development under high-osmotic conditions, where sexual development of wild type is blocked. This suggests that LsdA mediates the repression of the sexual cycle under these environmental conditions.

If RosA represses sexual development, the question is, How can we explain the drastic hyphal growth reduction and *fluffy* phenotype after overexpression? A possible answer is that this phenotype is caused by misregulation of different morphogenetic and metabolic pathways. On the other hand, it could be that growth and development reduction is a normal feature of RosA when induced by carbon depletion. Thus, a transient upregulation of *rosA* would prevent the immediate induction of the sexual cycle when inappropriate. During this short time, the metabolism could adapt to the starving situation, and the cell would start inducing, *e.g.*, enzymes for the mobilization of reserve material. The drastic overexpression phenotype does not allow any speculation about a role of RosA during late sexual development.

In addition to the transcriptional regulation of *rosA*, it appears that the protein activity also could be regulated post-transcriptionally. We found that RosA localized mainly in the cytoplasm and only some fraction of the protein accumulated in nuclei. In contrast, truncated versions of the protein with <294 aa were transported efficiently into the nuclei. This demonstrates that the predicted NLS is functional and suggests that nuclear import could be post-transcriptionally regulated, for instance, by changes in protein conformation to expose the NLS.

In summary, we show in this work that the induction of the sexual cycle in *A. nidulans* requires the integration of several environmental signals that might be transduced through different cascades and specific transcription factors. RosA could be involved in the transmission of signals, which integrate sexual development with growth under submersed, high-salt, or carbon-limited conditions. To further unravel the functions and the interplays of the transcription factors, it will be the challenge of future research to identify target genes for VeA, NsdD, and RosA.

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