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Fungal S-adenosylmethionine synthetase and the control of development and secondary metabolism in *Aspergillus nidulans*

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ABSTRACT

The filamentous fungus *Aspergillus nidulans* carries a single gene for the S-adenosylmethionine (SAM) synthetase SasA, whereas many other organisms possess multiple SAM synthetases. The conserved enzyme catalyzes the reaction of methionine and ATP to the ubiquitous methyl group donor SAM. SAM is the main methyl group donor for methyltransferases to modify DNA, RNA, protein, metabolites, or phospholipid target substrates. We show here that the single *A. nidulans* SAM synthetase encoding gene *sasA* is essential. Overexpression of *sasA*, encoding a predominantly cytoplasmic protein, led to impaired development including only small sterile fruiting bodies which are surrounded by unusually pigmented auxiliary Hülle cells. Hülle cells are the only fungal cell type which does not contain significant amounts of SasA. Sterigmatocystin production is altered when *sasA* is overexpressed, suggesting defects in coordination of development and secondary metabolism. SasA interacts with various metabolic proteins including methionine or mitochondrial metabolic enzymes as well as proteins involved in fungal morphogenesis. SasA interaction to histone-2B might reflect a putative epigenetic link to gene expression. Our data suggest a distinct role of SasA in coordinating fungal secondary metabolism and development.

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1. Introduction

S-adenosylmethionine synthetase (EC 2.5.1.6) is the only known enzyme that catalyzes the synthesis of S-adenosylmethionine (SAM) from methionine with ATP (Tabor and Tabor, 1984). SAM was discovered by Catoni (1953) and since then extensively investigated, especially in mammals as it is proposed to have therapeutic benefits in human diseases (Chiang et al., 1996). Besides ATP, the ubiquitous enzyme substrate SAM is one of the most frequently used substrates and the major methyl group donor in all living organisms. It is involved in methylation processes of DNA, RNA, proteins, metabolites, and phospholipids, in which the methyl group is transferred by a methyltransferase to the corresponding substrate (Mato et al., 1997). About 15 methyltransferase superfamilies have been identified and their classifications are based on substrate specificity rather than sequence similarities (Loenen, 2006).

SAM is the major cellular methyl donor but acts also as monoalkyl donor or 5'-deoxyadenosyl radical source. It is involved in a variety of processes in different organisms such as protection of

bacterial DNA, function of restriction enzymes, bacterial chemotaxis, regulation of gene expression, cellular differentiation, stress response, biosynthesis of secondary metabolites (e.g. polyketides or non-ribosomal peptides), virus latency, mRNA export, efficient translation and protection of mRNA integrity (Chiang et al., 1996). In addition, it is the sole carboxy-aminopropyl donor for the synthesis of polyamines, like spermidine. SAM is involved in the production of modified nucleotides in rRNA, or acts as transcriptional regulator by binding to riboswitches (Bjork et al., 1987; Bowman et al., 1973; Corbino et al., 2005; McDaniel et al., 2005; Winkler and Breaker, 2005).

SAM synthetases have been extensively studied in bacteria, yeasts, plants and mammals including humans (Cai et al., 1996; Peleman et al., 1989; Porcelli et al., 1988; Sakata et al., 1993; Thomas and Surdin-Kerjan, 1987), but less is known about them in filamentous fungi. SAM synthetases are highly conserved between bacteria and eukaryotes, with lower conservation in archaea (Graham et al., 2000). *Escherichia coli* or *Schizosaccharomyces pombe* possess one SAM synthetase, *Saccharomyces cerevisiae* and humans carry two isoforms, whereas there are even four isoenzymes in the plant *Arabidopsis thaliana* or the fly *Drosophila melanogaster*.

The catalytic reaction of SAM synthetase occurs in a two-step reaction. First, the triphosphate is cleaved from ATP and further hydrolyzed to PP_i and P_i before SAM is released (Mudd and Cantoni, 1958). The first crystal structure of the SAM synthetase MetK of

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E. coli was determined in 1996 (Takusagawa et al., 1996a). MetK consists of four identical subunits forming two dimers among which the active sites lie. The triphosphate moiety interacts extensively with the amino acid residues in the active site of the enzyme in order to cleave it at both ends, while the adenine and ribose moiety show weak interaction, what facilitates the release of SAM (Takusagawa et al., 1996b). After methyl group donation S-adenosylhomocysteine is formed, which is hydrolyzed further to homocysteine and adenosine.

Misregulation of the SAM synthetase encoding genes leads to changes in growth in several bacteria or the yeast *S. pombe*, and to changes in growth and secondary metabolism in the plant *A. thaliana*. In contrast, the filamentous fungus *Neurospora crassa* shows no phenotypical changes when expression of SAM synthetase encoding genes is changed (Belbahri et al., 2000; Hilti et al., 2000; Mautino et al., 1996; Newman et al., 1998; Ochi and Freese, 1982).

Aspergillus nidulans represents a model organism for filamentous fungi where development is coordinated to secondary metabolism. The life cycle is light-dependent. During illumination, primarily green asexual spores (conidia) are formed on conidiophores, whereas the sexual structures are repressed by light and primarily formed in dark and under limited oxygen levels (Adams et al., 1998; Bayram et al., 2010; Braus et al., 2010, 2002; Purschwitz et al., 2008). The closed sexual fruiting bodies (cleistothecia) are surrounded by nursing Hülle cells (Sarıkaya Bayram et al., 2010) and contain the red ascospores. Light is sensed by photoreceptors that transmit the signal to recipients like the velvet regulators. In the dark, the velvet protein VeA is transported together with the second velvet-like protein VelB into the nucleus, where they can form together with the putative methyltransferase LaeA the trimeric VelB–VeA–LaeA complex which coordinates sexual development and secondary metabolism (Bayram and Braus, 2011; Bayram et al., 2008). *A. nidulans* LaeA is required for secondary metabolite expression of the sterigmatocystin (ST) or penicillin clusters (Bok and Keller, 2004; Bok et al., 2006). An additional role of LaeA is its requirement for Hülle cell formation (Sarıkaya Bayram et al., 2010). LaeA carries a conserved SAM-binding site typical for nuclear protein methyltransferases (Bok and Keller, 2004; Kozbial and Mushegian, 2005) and there is accumulating evidence for an epigenetic control function of LaeA by chromatin remodeling (Reyes-Dominguez et al., 2010). A misregulation of the SAM synthetase encoding gene in *A. nidulans* might therefore affect development and secondary metabolism.

We analyzed the function of the single SAM synthetase SasA in the filamentous fungus *A. nidulans* for metabolism, growth, morphogenesis, development and secondary metabolism. We used genetic, cell biological and biochemical tools to obtain a comprehensive picture of the essential cellular function of SasA.

2. Experimental procedures

2.1. Strains and growth conditions

Strains used in this study are listed in Table S3. *A. nidulans* strains were cultivated on minimal medium (0.52 g l⁻¹ KCl, 0.52 g l⁻¹ MgSO₄, 1.52 g l⁻¹ KH₂PO₄, 0.1% trace element solution, pH 6.5, (Barratt et al., 1965) or on CMM medium (minimal medium with 0.1% casein hydrolysate) at 30 °C or 37 °C and supplemented appropriately with 1 µg ml⁻¹ pyridoxine–HCl, 1 µg ml⁻¹ uracil, 0.25 µg ml⁻¹ uridine, 1 µg ml⁻¹ pyrithiamine (Takara Bio Inc, Mönning, Germany), 120 µg·ml⁻¹ nourseothricin-dihydrogen sulfate (clonNAT, Werner BioAgents, Jena, Germany). 10 mM nitrate, ammonium or proline served as nitrogen source and 1% D-glucose as carbon source. For solid medium, 2% agar was added. For

vegetative growth, cultures were cultivated in light in submerged medium, for induction of asexual development, cultures were grown in light on solid medium, and for induction of sexual development, cultures were grown in dark on solid medium under oxygen limiting conditions (Clutterbuck, 1974). *E. coli* strains were propagated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl).

2.2. Strain construction and generation of transformation cassettes

Strains generated in this study are listed in Table S3. Plasmids and primers used in this study are given in Tables S4 and S5.

For overexpression strain AGB665, the nitrogen source driven promoter *P_{niiA}*, amplified from pME3160 with primers JG92/93, and *ptrA*, amplified from pPTRII with primers JG21/22, were fused via fusion PCR. The 1 kb upstream flanking region of *sasA* and 1.3 kb of *sasA* were amplified with primers JG83/150 and JG151/147 and fused to the *ptrA::P_{niiA}* cassette with primers JG84/152. The resulting cassette was subsequently cloned into strain TNO2A3 via homologous recombination.

For deletion of *sasA* the upstream and downstream flanking regions were amplified with primers JG47/48 and JG49/50 from genomic DNA and fused to *ptrA* with primers JG51/52. The deletion cassette was transformed into strain TNO2A3 via homologous recombination.

The C-terminally GFP- and TAP-tagged SasA strains AGB666 and AGB667 were generated by transformation via homologous recombination in strain TNO2A3. For both strains the 0.8 kb upstream flanking region and *sasA* were amplified with primers JG83/85 (GFP) or JG83/89 (TAP) and the 0.8 kb downstream flanking region was amplified with primers JG86/88 from genomic DNA. Subsequently, the amplicons were cloned to the *gfp::gpdA::nat^R* or *ctap::gpdA::nat^R* cassette, respectively, with primers JG84/87. For generation of cassettes, *gfp* was cloned to *P_{gpdA}::nat^R* (amplified from plasmid pNV1) with oligos OZG192/207 and *ctap* with OZG192/209. For generation of AGB668 and AGB669, strains AGB666 and AGB667 were crossed with wild type strain AGB154. For construction of the double *tap* strain *veA::ctap/velB::ctap* (AGB677), the strains *veA::ctap* (AGB273) and *velB::ctap* (AGB389) were crossed.

2.3. Transformation procedures

A. nidulans transformations were performed by polyethylene glycol mediated fusion of protoplasts as described previously (Eckert et al., 2000; Punt and van den Hondel, 1992).

2.4. Heterokaryon rescue

Heterokaryon rescue was performed according to the protocol by Osmani (Nayak et al., 2006). First, primary transformants of a standard *A. nidulans* transformation were picked and plated on selective and non-selective medium. Second, a primary transformant was excised together with agar, put on an agar plate with selective medium and grown 3 days at 30 °C. A fresh agar piece was cut out, put in liquid selective medium and grown for 3 days at 30 °C. Mycelia were harvested and genomic DNA extracted. Diagnostic PCR was performed with primers JG59/60.

2.5. Extraction of genomic DNA

Mycelia of vegetative grown cultures were ground in liquid nitrogen and lysed in appropriate buffer (50 mM Tris (pH 7.2), 50 mM EDTA, 30 g l⁻¹ SDS, 10 ml l⁻¹ β-mercaptoethanol) at 65 °C. Genomic DNA was extracted twice with phenol/chloroform (1:1 (v/v)) and subsequently precipitated with isopropanol and

50 mM sodium acetate (Kolar et al., 1988). Genomic DNA was dissolved in RNase treated EB buffer (Qiagen, Hilden, Germany) and stored at 4 °C.

2.6. Southern hybridization

Southern hybridization was performed according to standard protocols (Busch et al., 2003) with nonradioactively labeled probes (Amersham Bioscience, Buckinghamshire, UK). For probe preparations, DNAs were amplified from genomic DNA by PCR using primers listed in Tables S5 and S6.

2.7. Extraction of RNA

RNA was extracted from vegetative, asexual and sexual grown cultures. For vegetative cultures, the strain was grown 14 and 20 h in liquid medium at 37 °C. For asexual and sexual cultures, the strain was grown 20 h in liquid medium and the obtained mycelium was transferred to solid medium and grown 24, 48, and 72 h in light or dark at 37 °C. For Northern experiments, mycelia were ground in liquid nitrogen and mixed with Trizol (Invitrogen GmbH, Karlsruhe, Germany) and chloroform. After centrifuging, the upper phase was extracted twice with phenol/chloroform (1:1 (v/v)). Total RNA was precipitated with isopropanol overnight, dissolved in 0.1%-DEPC-water and stored at -20 °C. For quantitative real-time PCR, after grinding of mycelia in liquid nitrogen, total RNA was extracted with the RNEasy Plant Kit (Qiagen, Hilden, Germany).

2.8. Northern hybridization

Northern hybridization was performed according to the protocol of Brown and Mackey (1997). The gel was loaded with 10 µg RNA. DNA for probe was amplified from genomic DNA with primers JG147/151. The probe was labeled with [α -³²P]-dATP using the Prime-It[®]-II kit (Stratagene Europe, Amsterdam, Netherlands) (Feinberg and Vogelstein, 1983). Autoradiographs were produced using BioMaxMS films (Kodak Molecular Imaging, New Haven, CT, USA).

2.9. Quantitative real-time PCR

DNase digestion and subsequent cDNA synthesis was carried out with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) with 0.8 mg of RNA for each sample. Amplification was performed in a LightCycler 2.0 (Roche) with the RealMaster SYBR Rox Kit (5Prime) using 1 ml of the cDNA and *A. nidulans* primers JG385/386 and RT H2A 5'(+)/3'(-) (Table S5). The constitutively expressed H2A encoding gene was used for normalization and a sample without cDNA served as additional control. Amplification conditions were as follows: 36 cycles of 15 s at 95 °C, 22 s at 64 °C, 22 s at 70 °C, and an adjacent melting step (42–95 °C). The amount of *sasA* relative to histone H2A RNA was quantified using the DCT method with efficiency (Pfaffl et al., 2002). All real-time PCR experiments were performed in triplicate.

2.10. Fluorescence microscopy

A. nidulans strains (2000 spores) were inoculated in eight chambered borosilicate coverglass system (NUNC) supplemented with liquid medium or on glass slides covered with a thin layer of medium and incubated at 30 °C. Fluorescence photographs were taken with an AXIOVERT OBSERVER. Z1 (ZEISS) microscope equipped with a QUANTEM:512SC (PHOTOMETRICS) digital camera and the SLIDEBOOK 5.0 software package (INTELLIGENT IMAGING INNOVATIONS).

2.11. Protein extraction and Western blot

For isolation of proteins, crude extracts from vegetative grown cultures were prepared by grinding mycelia and extracting proteins with B300 buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 2 mM EDTA, pH 8) at 4 °C with addition of Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). After centrifugation, the supernatant was directly used for further analyses.

For Western experiments, the protein extracts were separated by PAGE and transferred onto a nitrocellulose membrane by electroblotting. As first antibody Anti-Calmodulin Binding Protein Epitope Tag (Biomol, Hamburg, Germany) and as second antibody goat-anti-rabbit IgG HRP (MoBiTec, Göttingen, Germany) was used. Prestained PageRuler (Fermentas GmbH, St. Leon-Rot, Germany) was utilized as marker. As detection reagent, the ECL technology (GE Healthcare Life Sciences, Munich, Germany) products were used.

2.12. Tandem affinity purification (TAP)

Vegetative cultures were grown over night at 30 °C in liquid medium and the mycelia were shifted to solid CMM medium (minimal medium with 0.1% casein hydrolysate) and grown under asexual or sexual inducing conditions. TAP experiments were performed as described previously (Bayram et al., 2008) with the TAP-tagged protein strain and the untagged wild type strain as control. In addition to the standard protocol, modified purifications were performed. For fixation of proteins, 28 ml l⁻¹ formaldehyde was added after 20 h of vegetative growth. After 20 min of incubation, 2.5 M glycine was added, and after 5 min, cultures were harvested as described before. A second modification included changes in buffer composition and concentration. Buffers were used without phosphatase inhibitors and instead of IPP300 the buffer IPP150 was used twice during washing steps. 4–8 µM S-adenosylmethionine was added to all buffers. In a shortened purification, directly after washing with IPP150, glycine buffer (0.1 M glycine, 150 mM NaCl, 0.1% Triton X-100, pH 2) was added. Proteins were eluted after 5 min of incubation and precipitated with trichloroacetic acid.

2.13. LC-MS/MS protein identification

Polyacrylamide gel pieces of stained protein bands were digested with trypsin according to Shevchenko et al. (1996). Tryptic peptides were extracted and identified using reversed-phase liquid chromatography coupled with an LCQ DecaXP mass spectrometer (ThermoElectron Corp., San Jose, CA) equipped with a nanoelectrospray ion source (Bayram et al., 2008).

2.14. Analysis of sterigmatocystin

For analysis of sterigmatocystin, plates were inoculated with 10⁷ spores and grown 3 days at 37 °C. An agar piece (Ø = 1.5 cm) was homogenized in 3 ml water and subsequently extracted with an equivalent volume of chloroform. The organic phase was evaporated and the residual metabolites were dissolved in 50 µl methanol. 15 µl of the extract was separated on TLC in acetone/chloroform 4:1 (v/v) and derivatized with an alcoholic aluminum chloride solution (20% (w/v)). Metabolites were visualized under λ = 366 nm. As standard, sterigmatocystin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used. The data were documented using CAMAG TLC Visualizer (CAMAG, Muttenz, Switzerland).

3. Results

3.1. *A. nidulans* genome contains one SAM synthetase gene with conserved motifs

In *A. nidulans* the SAM synthetase encoding gene AN1222 consists of 1278 nucleotides (with two introns in the N-terminal region) encoding a putative protein with 388 amino acids and a predicted molecular mass of 42.2 kDa. According to the *A. nidulans* nomenclature we named the gene as *sasA* (S-adenosylmethionine synthetase A). The deduced amino acid sequence of SasA exhibits two conserved motifs typical for all SAM synthetases (Fig. 1A). At the beginning of the central domain the hexapeptide GAGDQG is present, which binds the adenine moiety of ATP (Pajares et al., 1991). In the C-terminal domain the glycine-rich nonapeptide GGGAFSGKD is present. This motif forms a P-loop-like structure

and is proposed to be involved in binding the triphosphate of ATP (Takusagawa et al., 1996b).

A ClustalW alignment of the predicted protein sequence of SasA with other filamentous fungi, yeast, bacteria, plant, human and fly SAM synthetases revealed high similarities between the different species. The identity to other Aspergilli varies between 87% and 93%. Interestingly, all *Aspergillus* species carry only one SAM synthetase encoding gene, except for *Aspergillus flavus* and *Aspergillus oryzae*. These organisms possess two independent isoforms with only 55% identity to each other. They form a separate group in a phylogenetic comparison (Fig. 1B), indicating that these proteins have evolved to perform a different function. Likewise, *S. cerevisiae* possesses two independent SAM synthetase encoding genes *SAM1* and *SAM2*. However, the encoded proteins show a high amino acid identity of 91% to each other. In human, the identity between the two existing isoforms MAT1A and MAT2A is 84%. In *D. melanogaster*

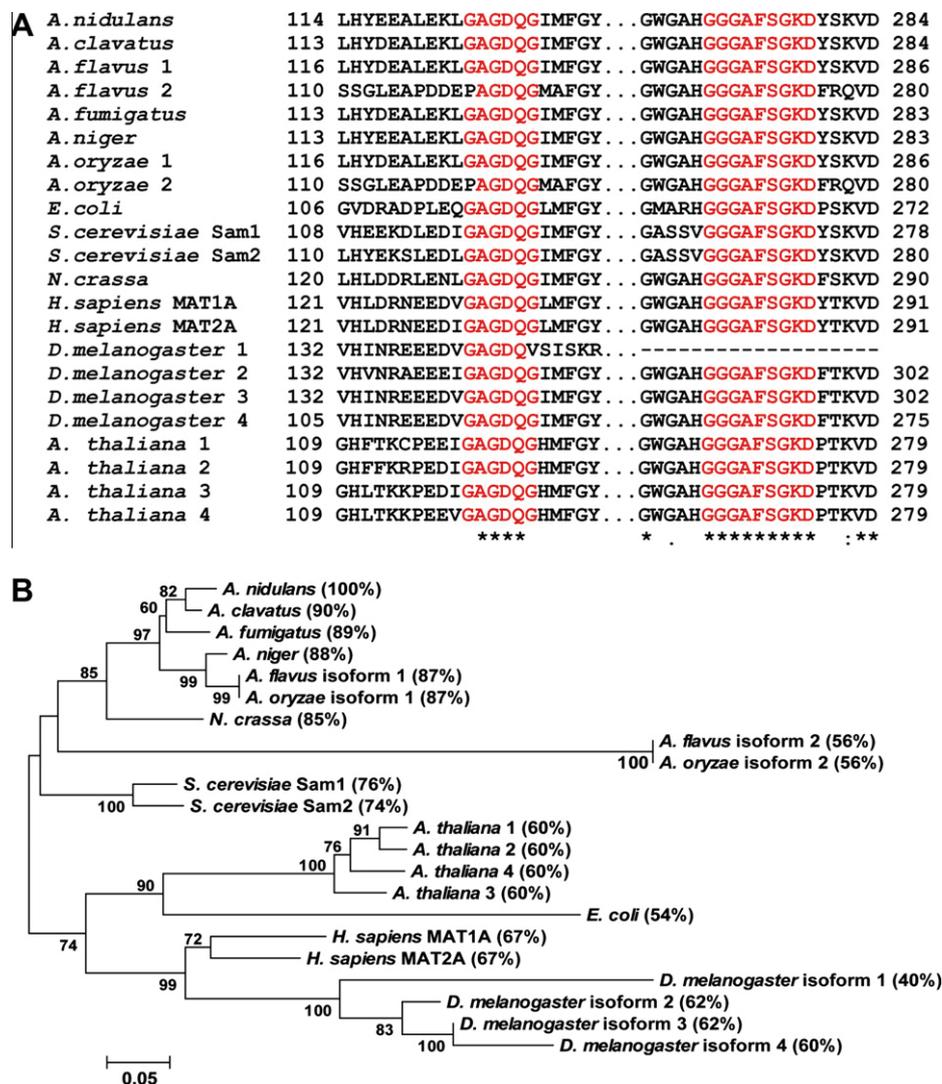


Fig. 1. Comparison of SAM synthetases from different organisms. (A) Protein comparison of SAM synthetases from different organisms. The conserved motifs are shown in red. GenBank Accession numbers for sequences are: *Aspergillus nidulans* (CBF87906), *Aspergillus clavatus* (EAW07774), *Aspergillus flavus* isoforms 1 and 2 (EED50079, EED51490), *Aspergillus fumigatus* (EDP56296), *Aspergillus niger* (CAK45307), *Aspergillus oryzae* isoforms 1 and 2 (BAE64158, BAE62029), *Escherichia coli* (CAH56923), *Saccharomyces cerevisiae* Sam1 and Sam2 (AAB67461, AAB64944), *Neurospora crassa* (AAC49260), *Homo sapiens* MAT1A and MAT2A (BAA08355, CAA48726), *Drosophila melanogaster* isoforms 1–4 (AAF51558, AAF51555, AAF51556, AAF51557), *Arabidopsis thaliana* isoforms 1–4 (AEE27438.1, AEE82085.1, AEC09311.1, AEE75948.1). (B) The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of amino acid differences per sequence. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Protein identities to *A. nidulans* SasA are given in parentheses.

four SAM synthetase isoforms exist. Isoforms 2–4 have identities of 93–97% to each other, and only the shorter isoform 1 differs highly with identities of 55–75% to isoforms 2–4.

The identity of *A. nidulans* SasA to the isoforms of *D. melanogaster* is 40–62%, the identity to the human isoforms 67%, to yeast Sam1 and Sam2 74–76%, to *E. coli* MetK 54%, and to the filamentous fungus *N. crassa* protein 85%. The evolutionary correlation is summarized in a phylogenetic tree (Fig. 1B). These data show that higher eukaryotes such as fly and human possess multiple copies of SAM synthetases, while fungi show variability in copy number between one and two.

3.2. The *sasA* gene is highly expressed during vegetative growth

We analyzed the expression of *sasA* during different developmental stages. Northern analysis was performed with RNA extracted from vegetative, asexual and sexual cultures grown for different time periods (Fig. 2A). For verification of the Northern results, we additionally performed real-time PCR (Fig. S1). The results show that *sasA* is strongly expressed during vegetative growth and that expression is downregulated during development in asexual or sexual grown cultures.

We compared the mRNA levels with the production of the SasA protein. A functional *tap*-tagged *sasA* strain was constructed (Fig. S2). Production of the tagged protein was monitored at the same stages of development as the transcripts by Western blot analysis with an anti-calmodulin binding protein antibody, recognizing the calmodulin binding peptide of the TAP-tag (Fig. 2B). Western results showed that SasA is strongly produced at vegetative stages and at early developmental stages, but production decreases during asexual and sexual development. Summarizing, mRNA expression and SasA protein production are correlating.

3.3. Constitutively expressed *sasA* is essential for the viability of *A. nidulans*

SAM is one of the most commonly used enzyme substrates and the major methyl group donor in all living organisms. Therefore, the deletion of the single *sasA* gene should be lethal, except there is a second, yet not identified SAM synthetase encoding gene in the genome of *A. nidulans*, which can compensate its function. We addressed whether *sasA* is essential by constructing a pyrithiamine resistance marker (*ptrA*) containing deletion cassette and integrated it into the genome of an *A. nidulans* wild type strain homologously (Fig. 3A). We obtained 18 primary transformants, which did not grow while generating single colonies.

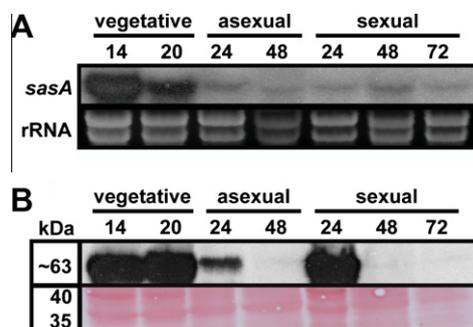


Fig. 2. Expression of *sasA* in *A. nidulans*. (A) Northern hybridization of *sasA* during vegetative, asexual, and sexual development. Cultures were grown for 14, 20, 24, 48, and 72 h. rRNA levels were used as loading control. (B) Western Blot analysis of C-terminally TAP-tagged SasA. Protein extracts were prepared after 14, 20, 24, 48, and 72 h of vegetative, asexual, and sexual growth. Ponceau staining is shown as loading control.

To proof whether *sasA* is essential, we applied the heterokaryon rescue technique (Nayak et al., 2006). First, a growth test on selecting and non-selecting medium was performed (Fig. 3B). The primary transformants grew on non-selecting medium, but they did not grow on selecting medium containing pyrithiamine, indicating the existence of a heterokaryon with a wild type *sasA* gene (*sasA*⁺, *ptrA*⁻) and the deletion (*sasA*⁻, *ptrA*⁺). Second, a diagnostic PCR was carried out using genomic DNA extracted from the heterokaryon (Fig. 3C). One primer annealing to the 5'-UTR region of the deletion cassette and one primer annealing in the genome directly behind the deletion cassette were chosen. The primer selection theoretically results in PCR products of 3.5 kb for the wild type (*sasA*⁺, *ptrA*⁻) and 4.2 kb for the deletion event (*sasA*⁻, *ptrA*⁺). The diagnostic PCR showed double bands at 3.5 and 4.2 kb for almost all clones, proving the existence of a heterokaryon. These results corroborate that the *sasA* gene is essential and its functions are indispensable for the cellular activities of *A. nidulans*.

3.4. Overexpression of *sasA* leads to sterile microcleistothecia with pigmented Hülle cells

We then addressed whether SasA production, which is primarily observed during vegetative development and at initial stages of fungal development, has a major impact on development of *A. nidulans*. We cloned *sasA* under the inducible *niiA/niiD* promoter. The correct homologous integration of the cloning cassette was verified by Southern hybridization experiments (Fig. S2). For verification of overexpression, the relative *sasA* mRNA abundance was determined by quantitative real-time PCR with primers for *sasA* cDNA under inducing conditions. The results show a 2.3-fold increase in *sasA* mRNA level compared to wild type mRNA (Fig. 4A).

For expression studies, wild type and *sasA* overexpressing (OE) strain were grown on different inducing and repressing media. Growth on nitrate medium induces the specific *niiA/niiD* transcription factor and thus *sasA* expression, whereas growth on the universal repressor ammonium reduces activity of the general nitrogen source transcription factor *areA* and therefore reduces *sasA* expression (Arst and Cove, 1973; Punt et al., 1995). Both strains were grown on inducing nitrate medium (NO₃⁻), inducing and repressing proline, nitrate and ammonium medium (proline + NH₄⁺ + NO₃⁻), repressing proline and ammonium medium (proline + NH₄⁺) and noninducing and nonrepressing proline medium. The developmental defects were analyzed during asexual and sexual growth.

During asexual development, *sasA* expression had impact on conidiation and colony diameter (Fig. 4B). While under inducing conditions (NO₃⁻), conidiation is rather similar to wild type, under inducing and repressing conditions (proline + NH₄⁺ + NO₃⁻) the amount of conidia is diminished to 25% of wild type level (Fig. 4C) and the colony diameter was reduced to 20% (Fig. 4D). As the *sasA* deletion results indicated, under repressing conditions (proline + NH₄⁺) the *sasA* mutant did not grow. Under noninducing plus nonrepressing conditions (proline) the mutant had a normal colony diameter, but produced nearly no conidia and additionally synthesized red pigments.

The sexual life cycle was drastically affected by *sasA* overexpression. The mutant was not able to perform its normal sexual life cycle. Under inducing conditions (NO₃⁻) the mutant strain generated very few and very small cleistothecia (microcleistothecia) with a diameter of 40–50 μm after 10 days of growth compared to normal wild type cleistothecia with a diameter of 100–200 μm (Fig. 5A and B). In addition, these microcleistothecia were sterile, lacking fertile ascospores. Concludingly, *A. nidulans* was not able to reproduce sexually when *sasA* was overexpressed.

Although only very few cleistothecia were developed, the amount of nursing Hülle cells was unaffected during sexual devel-

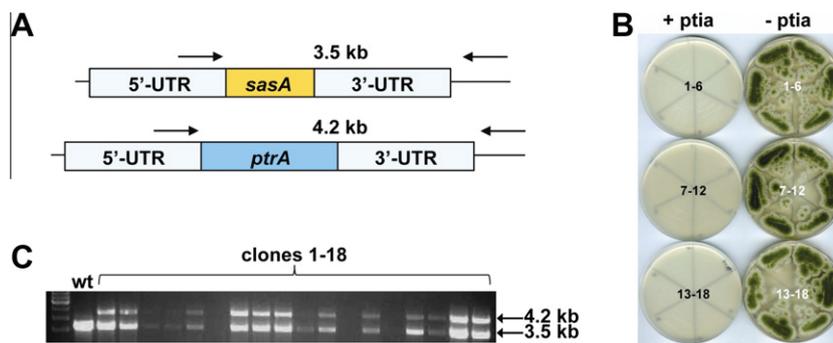


Fig. 3. Deletion of *sasA* is lethal. (A) Construction of *sasA* deletion. *sasA* was replaced by a pyrithiamine resistance marker (*ptrA*) via homologous recombination. Arrows indicate primers used for heterokaryon rescue PCR. For wild type locus a PCR amplicon of 3.5 kb and for deletion strain locus a PCR amplicon of 4.2 kb was expected. (B) Growth of primary transformants 1–18 was tested on selecting pyrithiamine (+ ptia) and non-selecting (– ptia) medium. (C) Diagnostic PCR with DNA extracted from primary transformants 1–18. Primers given in Fig. 3A were used. For wild type DNA, a band of 3.5 kb was detected, for clones 1–18 DNA, a double band of 3.5 kb and 4.2 kb was detected, proving the existence of a heterokaryon.

opment. The Hülle cells, which normally do not contain significant amounts of SasA (see Section 3.6), were remarkably different in comparison to wild type because they accumulated a pink colored pigment. Under inducing and repressing conditions (proline + NH_4^+ + NO_3^-) and under noninducing and nonrepressing conditions (proline) no sexual structures were formed and under repressing conditions (proline + NH_4^+) the mutant did not grow.

3.5. Overexpression of *sasA* leads to reduced production of sterigmatocystin

As overexpression of *sasA* had a possible impact on secondary metabolism indicated by the red color produced on proline medium (Fig. 4B), we analyzed whether overexpression influenced biosynthesis of the well-studied aflatoxin precursor sterigmatocystin (ST). Initially, we tested expression of genes for the transcription factor *afR* and the ketoreductase *stcU*, which are involved in ST biosynthesis, the master regulator of secondary metabolism *laeA* and the penicillin and terrequinone A producing *ipnA* and *tdiA* with Northern hybridization. Our results showed no significant changes in expression levels in *sasA* OE in comparison to wild type strain (data not shown).

We further analyzed whether there are posttranscriptional effects, which might influence secondary metabolism. Therefore, we extracted the non-polar metabolites from asexual grown wild type and *sasA* OE cultures and determined the amount of produced ST with thin-layer chromatography (TLC, Fig. 5C). Our results showed that production of ST in *sasA* OE strain is significantly reduced compared to wild type.

3.6. *SasA* is predominantly localized to the cytoplasm in most fungal cell types except Hülle cells

In *S. cerevisiae*, the SAM synthetases Sam1 and Sam2 are both primarily localized in the cytoplasm (Kumar et al., 2002). We determined the subcellular localization of the *A. nidulans* SasA via fluorescence microscopy. Therefore, *sgfp* (synthetic green fluorescent protein) was fused to *sasA* and integrated into the genome of *A. nidulans* homologously (Fig. S2). The construct was expressed under the native promoter of *sasA*.

Fluorescence microscopy revealed that SasA is primarily localized in the cytoplasm (Fig. 6A). SasA is not restrictively localized during asexual development to a distinct cell type but is visible in the cytoplasm of entire conidiophores including the conidiospores. During sexual development, SasA is localized to the ascospores but not to the Hülle cells, which are the nursing cells surrounding the cleistothecium (Sarıkaya Bayram et al., 2010).

Predominant cytoplasmic localization of SasA::GFP fusion indicates that SasA functions in the cytoplasm whereas any activity in other compartments might be only due to small SasA subpopulations.

3.7. Protein interaction studies revealed involvement of *SasA* in methionine metabolism and fungal growth

Protein interaction studies were performed for a more comprehensive picture of SasA function in the fungal cell. The tandem affinity purification (TAP) was applied using the SasA::TAP fusion strain (Fig. S2). The protein interactions were determined in extracts obtained from vegetative, asexual, or sexual grown cultures. In addition, an untagged wild type strain was analyzed under the same conditions as a control to exclude unspecific protein bindings. The purified protein extracts were separated on a SDS polyacrylamide gel. A protein band with an expected molecular weight for SasA of approximately 42 kDa was detectable (Fig. 6B).

During TAP analysis 22 putative interaction partners of SasA were identified via mass spectrometry (Table 1 and Table S1, ribosomal proteins were not listed). We further divided the identified proteins into subcategories. Three proteins (AN10474, AN4443, and AN8224) are connected to metabolism of the SAM synthetase substrate methionine. A second group spans proteins, which are putatively localized to the mitochondria in eukaryotes. An aconitase and a malate dehydrogenase were identified that are involved in citrate cycle. Additionally to the malate dehydrogenase, we found an aspartate aminotransferase. The respective homologous proteins in *S. cerevisiae* act together in malate–aspartate shuttle to transport NADH from cytoplasm into mitochondria (Easlon et al., 2008). The AN8216 homolog nucleoside diphosphate kinase Ynk1 of yeast, which catalyzes the transfer of gamma phosphates from nucleoside triphosphates to nucleoside diphosphates, is also partially localized to mitochondria in yeast (Amutha and Pain, 2003).

A third group of proteins putatively interacting with SasA are involved in gluconeogenesis. Phosphoenolpyruvate carboxykinase, catalyzing the GTP-dependent reaction of oxaloacetate to phosphoenolpyruvate, is a key enzyme in this reaction cascade. The fructose-bisphosphate aldolase catalyzes the conversion of fructose 1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. Gluconeogenesis is an important process as it allows organisms to grow on different carbon sources than sugar, like glycerol or ethanol (Haarasilta and Oora, 1975).

The fourth group comprises proteins, which are involved in fungal morphogenesis. This includes cell division, growth or cell wall growth. In this group are the conserved cytoskeletal elements tubulin and actin (Pruyne and Bretscher, 2000; Schatz et al.,

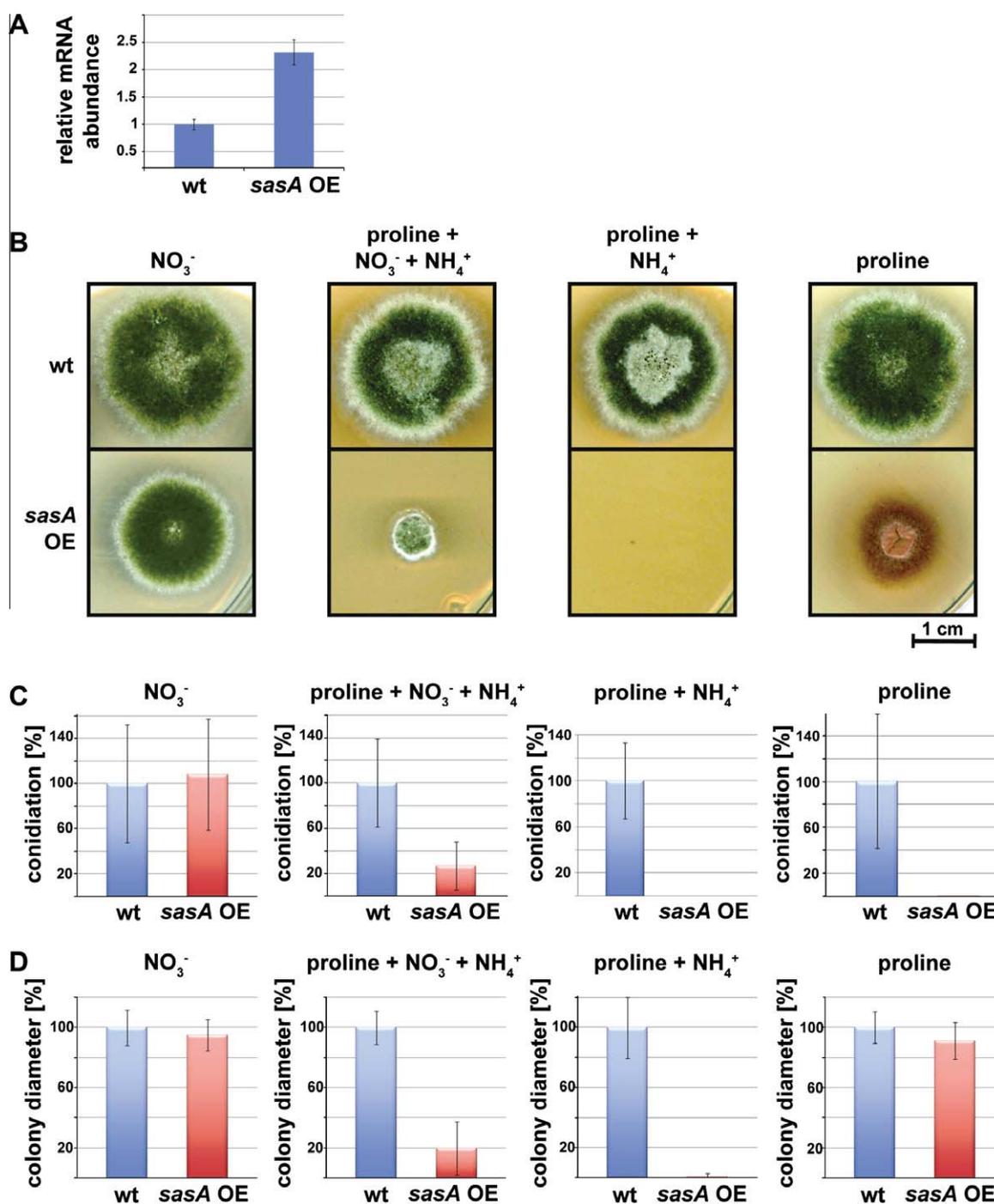


Fig. 4. Modified *sasA* expression in *A. nidulans*. (A) Relative mRNA levels of wild type and *sasA* OE strain measured by quantitative real-time PCR with primers specific for *sasA* cDNA. Cultures were grown in submerged cultures for 20 h at 37 °C. Normalization was carried out with primers for histone H2A. The delta CT method including efficiencies was used for quantification. (B) Growth test of *sasA* OE strain. *sasA* is under control of the inducible nitrate reductase promoter, which is induced by nitrate (NO_3^-), induced and repressed by medium containing proline, ammonium and nitrate (proline + NH_4^+ + NO_3^-), repressed by proline and ammonium (proline + NH_4^+) and noninduced and nonrepressed by proline. Strains were grown 3 days at 37 °C in light for analysis of asexual development. (C) Spore quantification of *sasA* OE compared to wild type. Wild type levels were set to 100%. (D) Colony diameter of *sasA* OE colonies compared to wild type. Wild type diameter was set to 100%.

1986), and calmodulin A which regulates many processes including response to various stress conditions, mating, budding, and actin-based processes (Cyert, 2001). The GTP-binding yeast protein Bud4 (homologous to AN6150) is involved in bud-site selection and required for axial budding pattern (Chant and Herskowitz, 1991; Sanders and Herskowitz, 1996) and Qri1 (homologous to AN9094) is important in e.g. cell wall biosynthesis (Cid et al., 1995).

Two proteins are putative stress proteins, like the heat shock protein AN10202 (homologous to Ssa1p in yeast), or the GABA transaminase (AN2248), which homologous protein in yeast is involved in oxidative stress response (Coleman et al., 2001). We identified histone-2B as the only histone which could be an interaction partner of SasA. Four additional putative SasA interacting proteins (other proteins) could not be obviously assigned to a specific category.

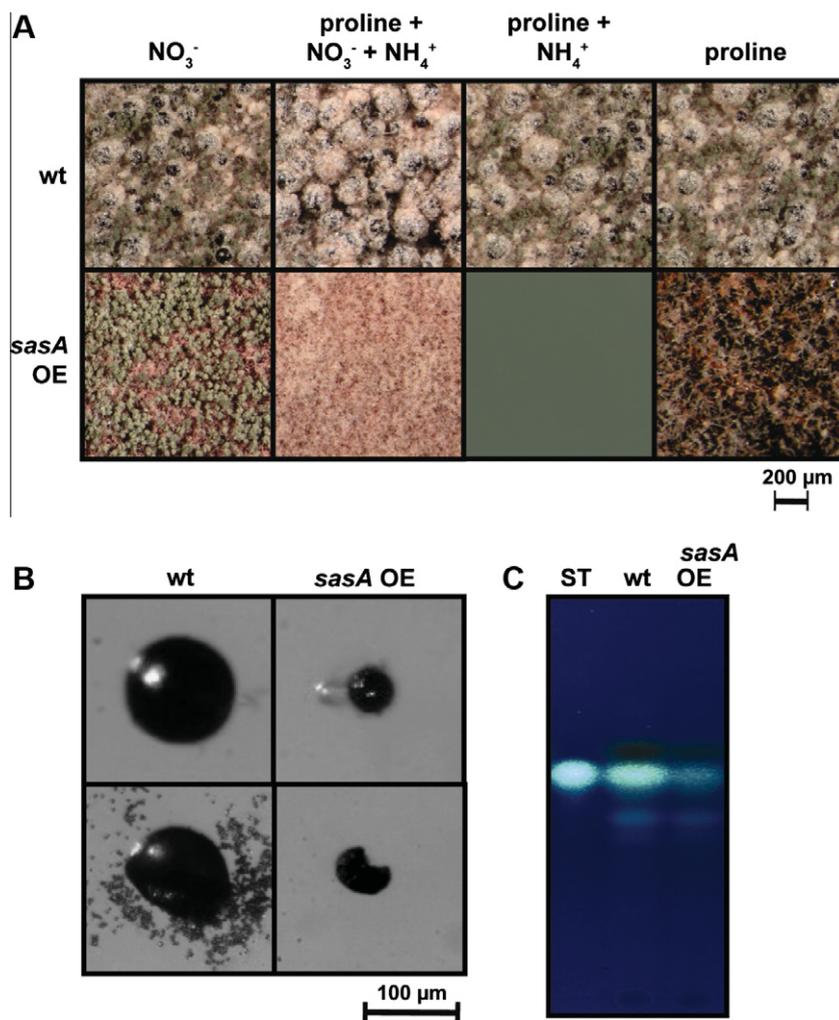


Fig. 5. Sexual development and secondary metabolism in *sasA* OE strain. (A) Phenotypes of wild type and *sasA* OE strains after 7 days of sexual development on nitrate (NO_3^-), proline, ammonium and nitrate (proline + $\text{NH}_4^+ + \text{NO}_3^-$), proline and ammonium (proline + NH_4^+) and proline containing medium. (B) Cleistothecia of wild type and *sasA* OE strain after 10 days of sexual growth on nitrate containing medium are shown. (C) Sterigmatocystin production levels of *sasA* OE and wild type were analyzed with TLC. Agar plates were inoculated with 10^7 spores and grown 3 days at 37 °C. Metabolites were extracted from defined agar pieces, separated on TLC (chloroform/acetone 4:1) and visualized with an alcoholic aluminum chloride solution at 366 nm. Sterigmatocystin (ST) was used as standard.

The putative methyltransferase LaeA is part of the trimeric velvet complex and coordinates sexual development and secondary metabolism. Sequence analysis showed that SasA possesses a SAM-binding motif (Bok and Keller, 2004). When a single subunit of the velvet complex was tagged the SasA protein could never be recruited. The velvet complex could only recruit SasA when a double tap-tagged *veA::ctap/velB::ctap* strain was used. Besides the already known interaction partners VosA and LaeA, we could also identify the SAM synthetase SasA as putative interaction partner of the velvet complex (Table S2). SasA was detected in a protein extract of sexual grown *veA::ctap/velB::ctap* cultures after 32 h of development, but not in the wild type control. Unfortunately, SasA could only be identified in one round of purification. These findings suggest a weak and presumably only transient interaction of SasA with the velvet complex.

4. Discussion

SAM synthetases catalyze the synthesis of the major methyl group donor SAM and represent highly conserved proteins which are present in one or more isoforms within a cell. SasA was analyzed in this study and represents the only SAM synthetase of

the filamentous fungus *A. nidulans*. The related *A. flavus* or *A. oryzae* possess two putative isoenzymes with only 55% amino acid identity, whereas the two *S. cerevisiae* isoenzymes are closely related with an identity of 91%. The single SAM synthetase gene *sasA* of *A. nidulans* is essential as it was also shown for the corresponding single *sam1* gene of the fission yeast *S. pombe* (Hilti et al., 2000). In organisms with two SAM synthetase encoding genes, like *S. cerevisiae*, the deletion of one gene can be compensated by the other, and both genes need to be deleted to achieve lethality (Cherest and Surdin-Kerjan, 1978).

Overexpression of *sasA* had a strong impact on development and secondary metabolism in the filamentous fungus *A. nidulans*. This was surprising because overexpression of the SAM synthetase encoding gene *ETH1* of the filamentous fungus *N. crassa* led to no phenotypical changes in growth rate or morphogenesis (Mautino et al., 1996). In the yeast *S. pombe*, low expression of *sam1* reduced growth, mating and sporulation, while overexpression led to methionine sensitive growth which could be partially suppressed by addition of adenine (Hilti et al., 2000). Misregulated SAM synthetase encoding genes also affect plants where overexpression and suppression of *Arabidopsis thaliana sam1* in tobacco led to yellow leaves, reduced plant height, necrotic lesions, and stunted plants with an altered secondary metabolism. Overexpression of

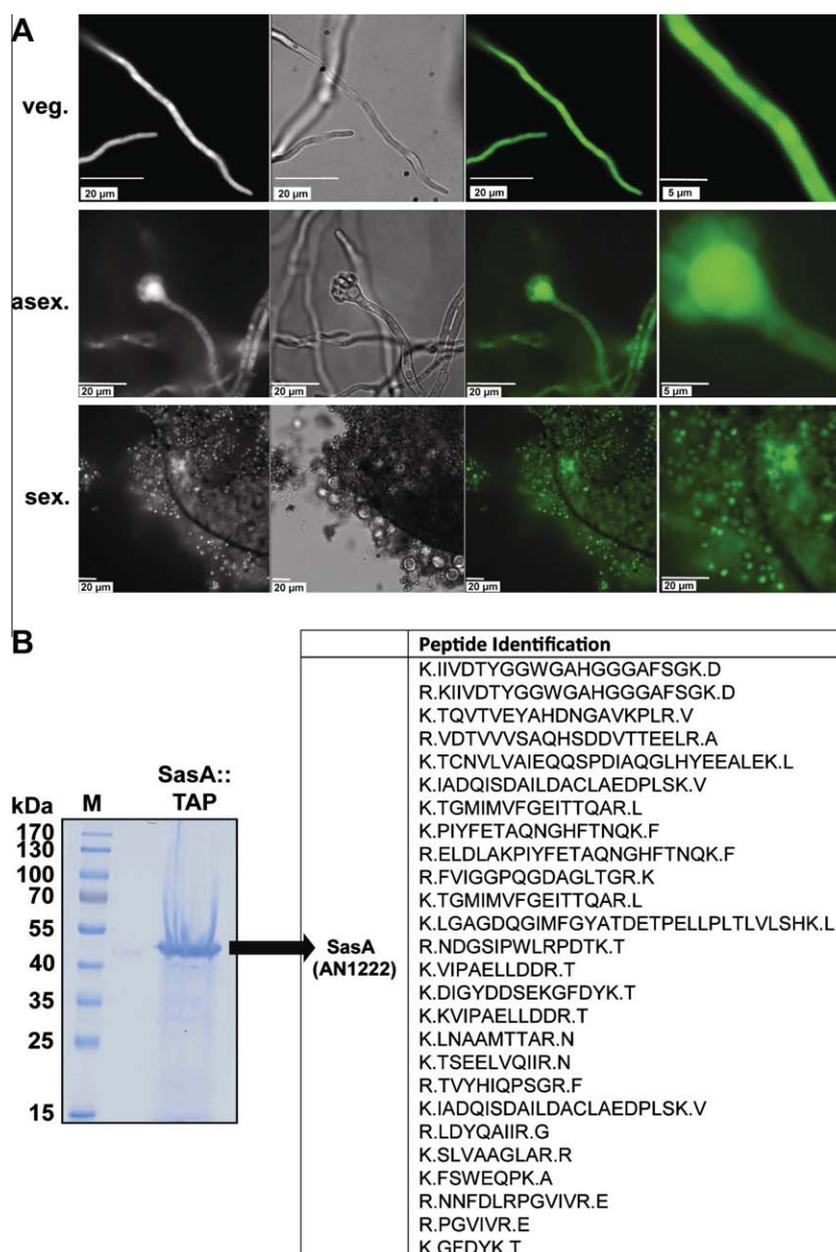


Fig. 6. Localization and protein interaction studies of SasA. (A) Localization of C-terminally GFP-tagged SasA was determined via fluorescence microscopy. Strains were cultivated in liquid medium for vegetative growth and on solid medium with and without light for asexual and sexual development, respectively. SasA is localized to the cytoplasm. (B) C-terminally TAP tagged SasA was purified via tandem affinity purification (TAP). SasA with a size of approximately 42 kDa was detected on SDS polyacrylamide gel. Peptides of SasA identified in mass spectrometry are given. A complete list of all detected proteins is given in Table S1.

sam1 enhanced nicotine and nor nicotine biosynthesis whereas reduced expression caused the production of volatiles as methanethiol (Belbahri et al., 2000; Boerjan et al., 1994). SAM synthetase overproduction in the bacterium *Bacillus subtilis* results in increased spontaneous sporulation (Ochi and Freese, 1982). Low expression of the SAM synthetase encoding *E. coli* gene *metK* resulted in the formation of filaments due to blocked septal ring formation (Newman et al., 1998; Wang et al., 2005). Only organisms which possess SAM feedback-inhibited SAM synthetases showed no overexpression phenotypes (Cabrero et al., 1988; Corrales et al., 1992, 1991; Duce et al., 1988; Markham et al., 1983; Thomas and Surdin-Kerjan, 1991; Yarlett et al., 1993). The corresponding *A. nidulans* or *S. pombe* enzymes are not feedback-regulated (Hilti et al., 2000; Pieniazek et al., 1973). Exogenously added methionine induced *sasA* expression in *A. nidulans* and

drastically increased the intracellular SAM levels in *S. pombe*. Feedback-inhibition might therefore serve as a protective mechanism in numerous organisms.

SasA interaction to other proteins was analyzed by tandem affinity purification (TAP), which allows identifying large relatively stable protein complexes (Bayram et al., 2008; Gavin et al., 2002). This might include proteins which only indirectly interact with SasA if several proteins of the same metabolic or developmental pathway form large complexes. SAM synthetases catalyze the reaction from methionine to SAM. This is consistent with the co-purification of SasA with proteins involved in methionine metabolism which might cooperate. Similarly, the *S. cerevisiae* glutamyl-tRNA synthetase Gus1 forms a complex with the methionyl- and glutamyl-tRNA synthetase cofactor Arc1 (Deinert et al., 2001; Galani et al., 2001). SasA interaction partners include presumably not only

Table 1
Putative interaction partners of *Aspergillus nidulans* SasA identified by tandem affinity purification (TAP). Proteins were purified from vegetative (V), asexual (A), and sexual (S) samples. Homologous proteins in *Saccharomyces cerevisiae* are given.

<i>A. nidulans</i>	<i>S. cerevisiae</i>	Protein description	Dev.
<i>Methionine metabolism</i>			
AN10474	Arc1	Cofactor for methionyl- and glutamyl-tRNA synthetases	A, S
AN4443 (Meth)	Met6	Cobalamin-independent methionine synthase	A, S
AN8224	Gus1	Glutamyl-tRNA synthetase	A, S
<i>Putative mitochondrial proteins</i>			
AN1993	Aat1	Aspartate aminotransferase	A, S
AN5525 (AcoA)	Aco1	Aconitase	V, S
AN6717	Mdh1	Malate dehydrogenase	V, A, S
AN8216	Ynk1	Nucleoside diphosphate kinase	V, S
<i>Gluconeogenesis</i>			
AN1918 (AcuF)	Pck1	Phosphoenolpyruvate carboxykinase	A, S
AN2875	Fba1	Fructose 1,6-bisphosphate aldolase	A
<i>Fungal morphogenesis</i>			
AN0316	Tub1	α -Tubulin	V, A
AN2047	Cmd1	Calmodulin	V
AN6150	Bud4	GTP-binding protein	V, A
AN6542	Act1	Actin	V, A
AN7254	Cdc48	Cell division control protein 48	A, S
AN9094	Qri1	UDP-N-acetylglucosamine pyrophosphorylase	A, S
<i>Stress</i>			
AN10202	Ssa1	ATPase, member of heat shock protein 70 family	V, A, S
AN2248	Uga1	γ -Amino-N-butyrate (GABA) transaminase	A, S
<i>Histone</i>			
AN3469	Htb1/Htb2	Histone-2B	V, A
<i>Other proteins</i>			
AN10540	Yol057w	Dipeptidyl-peptidase III	A, S
AN4739	Ade1	N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase	S
AN5989	–	NAD-dependent epimerase/dehydratase	A, S
AN7708	–	Aldo-keto reductase	A, S

methylation targets but also associated proteins. Future bimolecular fluorescence complementation (BiFC) or yeast-two-hybrid studies will discriminate between direct and indirect SasA interaction partners listed in Table 1.

Similar to yeast, SasA of *A. nidulans* is primarily localized to the cytoplasm, whereas mitochondrial or nuclear subpopulations are significantly less pronounced. A yeast mitochondrial carrier protein Sam5 transports SAM from cytoplasm to mitochondria, where it is needed for biotin biosynthesis (Marobbio et al., 2003). SasA interaction candidates include mitochondrial proteins involved in citrate cycle or in the malate-aspartate shuttle. It is currently unclear whether the interaction of these proteins with SasA occurs outside of the mitochondria or reflects small mitochondrial SasA subpopulations.

mRNA and protein levels of SasA are high during vegetative growth and early development of *A. nidulans*. The identified interaction partners (Fig. 7) suggest multiple cellular roles of SasA. Early stages of development require changes in morphogenesis and an increased cell division rate. The developmental overexpression phenotypes including colony diameter, spore production and viability suggest that either vegetative and early developmental production of SasA or changes in the availability of the co-factor SAM can affect fungal morphogenesis.

This is further supported by the identified putative SasA interaction partners. As found for *A. nidulans*, yeast Sam1 also recruits proteins required for morphogenesis like the aconitase Aco1 or α -tubulin Tub1 (Gavin et al., 2006). In addition, the recruitment of the cobalamin-independent methionine synthetase Met6 (Malkowski et al., 2007) and the heat shock protein Ssa1 (Gong et al., 2009) seem to be conserved. Heat shock proteins are methylated in response to stress (Chiang et al., 1996). Yeast Aco1 interacts with calmodulin Cmd1 (Krogan et al., 2006), which itself interacts with actin Act1 (Collins et al., 2007) and the nucleoside diphosphate kinase Ynk1 (Ho et al., 2002). All these data support that yeast Sam1

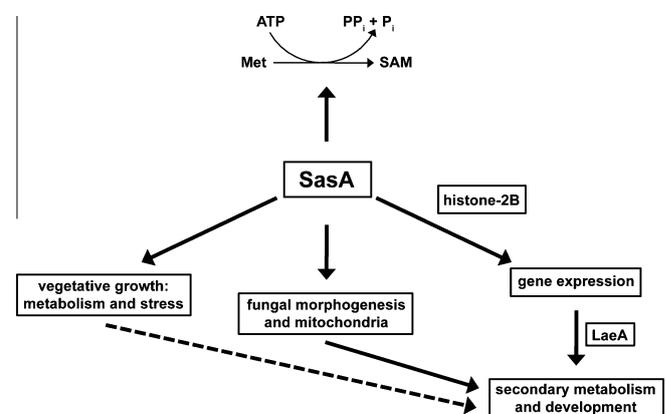


Fig. 7. Proposed model for SasA actions in *Aspergillus nidulans*. SasA catalyzes the biosynthesis of SAM from methionine (Met) and ATP. Expression of SasA has impact on metabolism and stress response during vegetative growth, on fungal morphogenesis including spore production, colony diameter and sexual fruiting body formation, and on mitochondrial processes. Additionally, it might be involved in coordination of development and secondary metabolism by regulating gene expression.

and *A. nidulans* SasA seem to (directly or indirectly) interact with multiple protein partners.

SAM-dependent methyltransferases were not detected as possible interaction partners of SasA by TAP purification. SAM synthetase interactions with methyltransferases were also not detected in the yeast proteome (Gavin et al., 2006). The putative methyltransferase LaeA possesses a conserved SAM binding motif which is typical for nuclear protein methyltransferases (Bok and Keller, 2004). LaeA is part of the VelB–VeA–LaeA complex which coordinates development and secondary metabolism. Single TAP-tagged VelB or VeA did not recruit SasA, but a strain where both velvet

domain proteins were TAP-tagged could recruit SasA (Table S2). A possible explanation might be a transient interaction between SasA and the VelB–VeA–LaeA complex which might explain why *sasA* overexpression results in defects in the coordination of secondary metabolism and development. Additional experiments need to be performed to verify the putative interaction. Consistently, *sasA* overexpression also affects Hülle cell formation, which is controlled by LaeA. Overproduction of SasA might affect LaeA function and subsequently methylation of histone-2B and secondary metabolite production (Fig. 7). Histone-3 trimethylation at lysine 9 had been found to be associated with the repression of secondary metabolism clusters in *A. nidulans*. LaeA is presumably involved in reversal of this heterochromatin signature inside the cluster (Reyes-Dominguez et al., 2010). The interaction of SasA with histone-2B is consistent with the finding that histone-2B can stimulate histone methyltransferase activity when modified at lysine 34 by ubiquitin (Wu et al., 2011). Future studies will elucidate whether LaeA epigenetic control includes not only the modulation of the histone-3 but also the histone-2B signature.

5. Conclusions

We present the first comprehensive study of the SAM synthetase SasA of the filamentous fungus *A. nidulans*. Our results support a complex SasA network of the major methyl group donor synthetase. SasA seems to be part of several protein complexes including methylation targets and associated proteins. SasA is involved in a variety of cellular processes and a precise balance of SasA levels is necessary to enable normal fungal morphogenesis, development and secondary metabolism. SasA contributes to methionine and other metabolic pathways, stress response, fungal morphogenesis and secondary metabolism. An interaction of SasA to histone-2B supports a SasA mediated link to epigenetic control by methylation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fgb.2012.04.003>.

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