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Microarray and real-time PCR analyses reveal mating type-dependent gene expression in a homothallic fungus

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Abstract *Sordaria macrospora* is a homothallic ascomycete which is able to form fertile fruiting bodies without a mating partner. To analyze the molecular basis of homothallism and the role of mating products during fruiting body development, we have deleted the mating type gene *Smta-1* encoding a high-mobility group domain (HMG) protein. The Δ *Smta-1* deletion strain is morphologically wild type during vegetative growth, but it is unable to produce perithecia or ascospores. To identify genes expressed under control of *Smta-1*, we performed a cross-species microarray analysis using *Neurospora crassa* cDNA microarrays hybridized with *S. macrospora* targets. We identified 107 genes that are more than two-fold up- or down-regulated in the mutant. Functional classification revealed that 81 genes have homologues with known or putative functions. Comparison of array data from Δ *Smta-1* with those from three phenotypically similar mutants revealed that only a limited set of ten genes is deregulated in all mutants. Remarkably, the *ppg2* gene encoding a putative lipopeptide pheromone is 500-fold down-regulated in the Δ *Smta-1* mutant while in all other sterile mutants this gene is up-regulated.

Keywords *Sordaria macrospora* · *Neurospora crassa* · Mating type · Fruiting body development · Cross-species microarray hybridization · Differential gene expression

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Introduction

The regulation of sexual reproduction is one of the central processes in the life cycle of most eukaryotes. Like sex chromosomes in animals and plants, the allele type at the fungal mating type locus determines sexual compatibility between haploid individuals in fungi (Fraser and Heitman 2004, 2005). In ascomycetous fungi, alternative versions of the mating type locus on homologous chromosomes are completely dissimilar and encode unrelated proteins (Coppin et al. 1997; Shiu and Glass 2000). They have thus been termed idiomorphs instead of alleles (Metzenberg and Glass 1990). The genetic breeding mechanism of fungi in which sexual reproduction occurs only between strains of opposite mating type is called heterothallism.

The mating system of *Neurospora crassa* is the best characterized of any heterothallic filamentous ascomycete. The idiomorphs conferring mating behavior of the partners are designated *mat A* and *mat a* (Glass et al. 1988). The *A*-idiomorph contains three genes: *mat A-1*, *mat A-2* and *mat A-3* (Ferreira et al. 1996; Glass et al. 1990a). The MAT A-1-polypeptide, which is the major regulator of sexual development in *A* strains, accommodates a DNA-binding motif that shows similarity to the *Saccharomyces cerevisiae* *mat α 1* mating-type protein. The *N. crassa* genes *mat A-2* and *mat A-3* encode a protein without characteristic DNA-binding motif and a protein with a high mobility group (HMG) domain, respectively (Ferreira et al. 1998). The HMG motif is found in several DNA-binding proteins (Grosschedl et al. 1994).

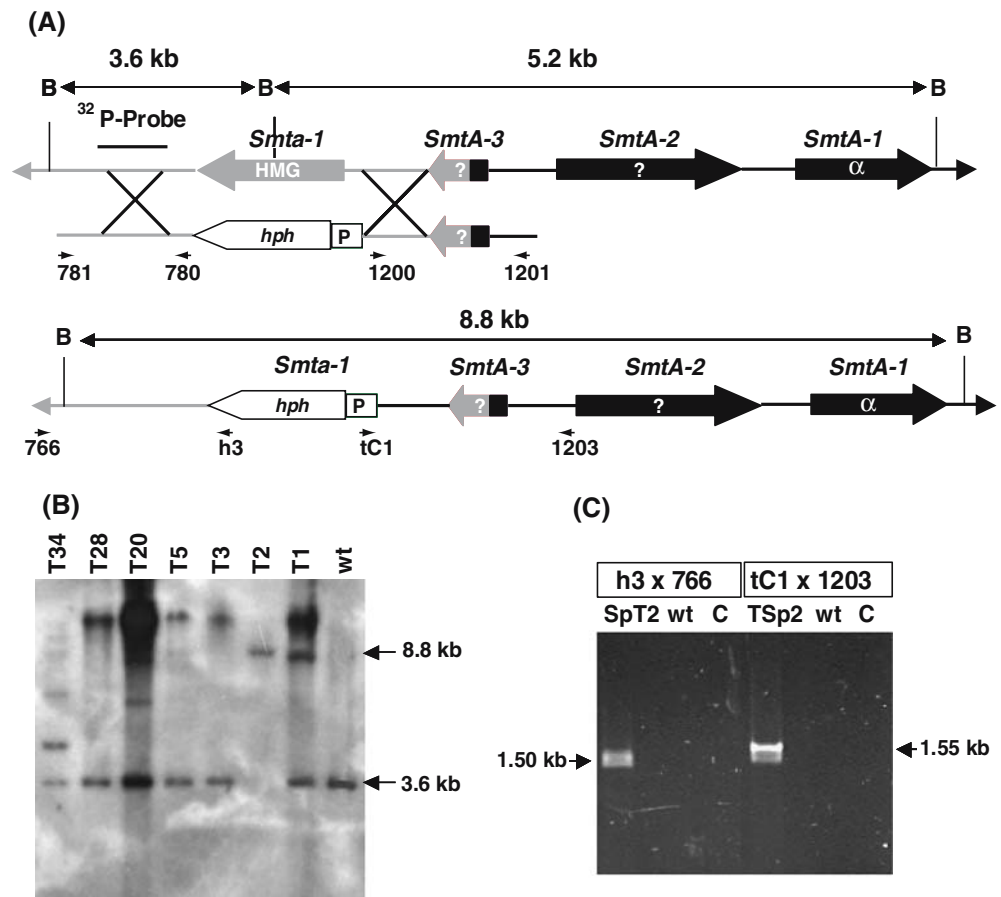
The *a*-idiomorph contains two genes, *mat a-1* and *mat a-2*. The *mat a-1* gene encodes a HMG domain protein that is the major regulator of mating in *a* strains, whereas *mat a-2* codes for a protein of unknown function (Pöggeler and Kück 2000; Staben and Yanofsky 1990).

In addition to heterothallism, a second mating system, designated homothallism, can be observed in

ascomycetes. Homothallic species have no genetically definable mating-type and are self-fertile. The mycelium derived from a uninucleate ascospore or a vegetative spore of a homothallic fungus is able to complete the sexual cycle without the interaction with a mating partner (Pöggeler 2001). *Sordaria macrospora* is a homothallic ascomycete that is closely related to the heterothallic *N. crassa*. An analysis of the mating-type locus of *S. macrospora* revealed that this locus contains sequences homologous to both the *mat a* and *mat A* idiomorphs of *N. crassa* (Fig. 1) (Pöggeler et al. 1997). In the mating-type locus of *S. macrospora*, four different ORFs were identified, *Smt a-1*, *Smt A-3*, *Smt A-2*, and *SmtA-1*, all of which are transcribed (Pöggeler and Kück 2000). Two of these genes (*SmtA-1* and *Smta-1*) contain domains typical for eukaryotic transcription factors (Fig. 1). When expressed in haploid strains of both mating types of the related heterothallic *Podospira anserina*, the mating-type locus of *S. macrospora* can induce fruiting body formation (Pöggeler et al. 1997). Furthermore, we previously demonstrated that SMTA-1 and SMTa-1 are able to form a heterodimer and are able to activate transcription of reporter genes in yeast. The mating-type proteins, SMTa-1 and SMTA-1, thus contain properties characteristic for mating-type-encoded transcriptional regulators of other ascomycetes (Jacobsen et al. 2002).

In the yeast *S. cerevisiae*, pheromone signaling was shown to enable cells of opposite mating type to detect each other. Here, the unique proteins encoded by the two different mating-type loci directly act as transcriptional regulators on mating-type specific expression of pheromone and pheromone receptor genes (Bardwell 2005; Herskowitz 1989). Interestingly, genes encoding two different pheromone precursors and two pheromone receptors have not only been found in the heterothallic *N. crassa*, but also in the homothallic *S. macrospora* (Bobrowicz et al. 2002; Pöggeler 2000; Pöggeler and Kück 2001). The pheromone-precursor gene *ppg1* is predicted to encode an α -factor-like peptide pheromone and the *ppg2* gene an a-factor-like lipopeptide pheromone (Pöggeler 2000). The deduced gene products of *pre1* and *pre2* show structural similarities to the yeast a-factor receptor Ste3p and α -factor receptor Ste2p, respectively (Pöggeler and Kück 2001). As in the yeast *S. cerevisiae*, the expression of pheromone genes and pheromone-receptor genes in filamentous ascomycetes is supposed to be controlled directly by transcription factors encoded by the mating-type genes (Debuchy 1999). In the heterothallic *N. crassa*, *mat A* or *mat a* mating-type specific transcription factors are present in *mat A* or *mat a* strains, respectively, and it was demonstrated that pheromone-encoding genes of *N. crassa* are predominantly expressed in a mating-type-specific manner

Fig. 1 Deletion of the *S. macrospora Smta-1* gene. **a** Structure of the *Smta-1* genomic region and construction of a $\Delta Smta-1$ deletion strain. Positions of primers used to amplify the deletion construct and verify the homologous recombination at the mating type locus are indicated. *P*, *A. nidulans trpC* promoter; *hph*, *E. coli* hygromycin resistance gene; *B*, *Bam*HI. **b** Southern analysis. Genomic DNA from *S. macrospora* wild type (wt) and seven transformants (T1, T2, T3, T5, T20, T28 and T34) was digested with *Bam*HI, separated on a 1% agarose gel, blotted and hybridized with the 840 bp-³²P probe indicated in (a). **c**. PCR analysis for verification of homologous recombination from a single spore isolate SpT2 (S59403) of primary transformant T2 and the wild type. Positions of primers are indicated in (a)



(Bobrowicz et al. 2002). On the contrary, both types of mating-type regulators are expressed in the same individual of the homothallic ascomycete *S. macrospora* (Pöggeler et al. 1997a). It is thus not surprising that both pheromone-precursor genes and pheromone-receptor genes of *S. macrospora* were shown to be transcriptionally expressed during sexual development in the same individual (Pöggeler 2000; Pöggeler and Kück 2001).

To further determine the functionality of the *S. macrospora* mating-type genes, we deleted the *Smta-1* gene encoding a HMG protein, analyzed the phenotype of the $\Delta Smta-1$ mutant, and examined the effect of the deletion on the expression of pheromone genes. The high degree of sequence identity between *S. macrospora* and *N. crassa* allowed us to perform cross-species microarray hybridizations using *N. crassa* cDNA microarrays for hybridization with targets prepared from *S. macrospora* (Nowrousian et al. 2004, 2005). Using this cross-species microarray technology, we have identified 107 genes that are at least twofold up- or down-regulated in the $\Delta Smta-1$ mutant.

Materials and methods

Strains and culture conditions

Cloning and propagation of recombinant plasmids was done under standard conditions (Sambrook et al. 2001). *E. coli* strain SURE was used as the host for plasmid amplification (Greener 1990). The *S. macrospora* strain K (S17736) has a wild-type phenotype and was derived from our laboratory collection (Department of General and Molecular Botany, Bochum, Germany). The wild-type strain and the *S. macrospora* knockout mutant $\Delta Smta-1$ and the complemented strain $\Delta Smta-1 + Smta-1$ were cultivated on corn-meal medium or CM medium (Esser 1982; Nowrousian et al. 1999). *S. macrospora* strains used for the isolation of RNA were grown as described previously (Nowrousian et al. 2005).

Construction of the knockout plasmid

Deletion of the *S. macrospora* mating type gene *Smta-1* was achieved as follows. The 5'- and 3'- regions of the *Smta-1* gene were amplified by PCR from genomic DNA of a wild-type *S. macrospora* strain using oligonucleotide pairs 780/781 and 1200/1201 as primers (Fig. 1). The sequences of all PCR primers used in this work are summarized in Table 1. Amplification with 1200 and 1201 generated *ApaI* sites at the ends. The *Smta-1*-fragment (1,448 bp) containing the 5'-region of the *Smta-1* gene was inserted into vector pDrive (Qiagen, Hilden, Germany) resulting in plasmid *psaI*prom1. Plasmid *psaI*term was generated by cloning the 1,523 bp 3'-*Smta-1*-fragment into pGEM-T (Promega, Mannheim, Germany). The 1.5 kb-*ApaI* fragment encoding

Table 1 Oligonucleotide primers used in this investigation

Oligo	Sequence 5' > 3'
766	CCATCCGCGACAAGGAGACA
780	CCCGGTCACCAGGGGTATCACC
781	TGGAGCCAGTACTGGAGTTG
1200	GGGCCCGCGATGTAGACGGAGGAATGT
1201	GGGCCCTTGAGGGACATCGCATAACCAC
1203	CCTGTGGGGTATTACATGCCA
h3	ACTCGTCCGAGGGCAAAGGAATAG
tC1	GATCCGCCTGGACGACTAAACC
ppg1-f	AAGCGCCAGGTTGACGAGC
ppg1-r	CGTCCTTCTTCTCCTCCTCGGT
ppg2-f	CGGTATCTCGCCTCTCAACGT
ppg2-r	GTTGTGCTCCCATGTGCAGA
pre1-f	GCATTACGCCACATCAAC
pre1-r	GTTGTGCCGAAGGTGATGCA
pre2-f	TCCACCCGTTCCATACCCTG
pre2-r	TCGATGCAAGCTAGTTCCGG
SSU-f	ATCCAAGGAAGGCAGCAGGC
SSU-r	TGGAGCTGGAATTACCGCG

the 3'-*Smta-1*-region was then inserted into the *ApaI* site of plasmid *psaI*prom1. The resulting plasmid *psaI*-1 contains the 5'-region of the *Smta-1* gene adjacent to the 3'-region of the gene. The two sequences are separated by a single *SalI* restriction site. Next, the 1.4 kb *SalI hph* cassette of pCB1003 (Carroll et al. 1994) was inserted into the single *SalI* site of plasmid *psaI*-1. The resulting plasmid *psaI*hyg-2 was used as a template to amplify the *Smta-1-hph* cassette with oligonucleotides 781/1201 as primers. The 4.4 kb PCR fragment obtained was transformed into *S. macrospora* wild-type strain to generate a knockout of the *Smta-1* gene by homologous recombination (Fig. 1).

Transformation of *S. macrospora* and crosses

Transformation of *S. macrospora* was performed according to Nowrousian et al. (1999). Fungal protoplasts were transformed with linear PCR fragments amplified from plasmid *psaI*hyg-2. Transformants were selected on hygromycin B-containing medium (110 U/ml).

Genomic DNA was isolated as described previously (Pöggeler et al. 1997). Transformants were screened for homologous integration (Fig. 1) by Southern blot analysis. Genomic DNA from 45 hygromycin-resistant transformants was subjected to Southern blot analysis after digestion with *Bam*HI. Among these, one strain lacking wild-type nuclei (corresponding to a 3.6 kb hybridizing fragment), but carrying $\Delta Smta-1$ nuclei (8.8 kb hybridizing fragment) was identified (strain T2, Fig. 1b). Successful homologous recombination was confirmed by PCR amplification. Primers 766 and h3 were used for the 5'-flanking region and primers tC1 and 1203 for the 3'-flanking region of the *Smta-1* gene. Fungal transformants are often heterokaryotic, i.e. mycelia carry transformed and non-transformed nuclei. Therefore, homokaryotic single spore isolates of

primary transformants carrying the *Smta-1* knockout were created by crossing the primary transformant and a spore color mutant *fus1-1* and selecting the hygromycin-resistant progeny. The single spore isolate S59403 was used for further analyses.

For complementation of mutant defects, strain S59403 was transformed with cosmid vector D9 containing the mating-type locus of *S. macrospora* (Pöggeler et al. 1997). Since strain S59403 is already hygromycin-resistant, cosmid D9 was used in cotransformation with plasmid pNat1 containing the nourseothricin-coding gene *nat1*. The *nat1* gene provides a selectable marker for fungal protoplasts in transformation experiments (Krugel et al. 1993). In plasmid pNat1, *nat1* is expressed under the control of the *gpd* promoter and *trpC* terminator of *A. nidulans* (Kück and Pöggeler, unpublished). Transformants were selected on nourseothricin-containing medium (50 µg/ml).

Preparation of nucleic acids and hybridization protocols

DNA isolation was performed as described by Pöggeler et al. (1997). RNA and poly(A) RNA was prepared as described previously (Nowrousian et al. 2005). The integrity of RNAs was verified by agarose gel electrophoresis and Northern blot analysis prior to extraction of poly(A) RNA. Southern and Northern blotting were performed and hybridized according to standard techniques (Sambrook et al. 2001) using ³²P-labeled DNA probes.

Microarray hybridization

The *N. crassa* microarrays used in these experiments were prepared from two unigene libraries described previously (Dvorachek et al. 2001; Nowrousian et al. 2003). Microarray hybridization with Cy3- and Cy5-labeled probes were performed as described by Nowrousian et al. (2005). For analysis of the Δ *Smta-1* mutant, two independent experiments were carried out; in the first, the mutant cDNA was labeled with Cy3 and the wild type with Cy5; in the second experiment, the dyes were switched. Analyses of TIFF-files from arrays and normalization were performed as described previously (Nowrousian et al. 2005). Clones were defined as regulated differentially in the mutant if they fulfilled one of the following criteria: (1) mutant/wild type expression ratio < 0.5 or > 2 in the two independent experiments; (2) the mean ratio of transcript levels of the mutant versus the wild type from two independent experiments was < 0.5 or > 2 with a coefficient of variance < 0.5.

Quantitative real-time PCR

For quantitative real-time PCR (RT-PCR), reverse transcription of 1 µg of total RNA was performed as

described previously (Nowrousian et al. 2005). Real-time PCR was performed in a DNA Engine Opticon 2 (MJ Research) with qPCR MasterMix for SybrGreen (Eurogentec) in a volume of 20 µl. Each reaction was carried out in triplicate with each primer at 0.3 µM (Table 1). Primer pairs were selected to have melting temperatures of 61–62°C and to yield amplicons of 120–150 bp. PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis. Amplicon size for each primer pair was verified by gel electrophoresis. Mean Ct values (threshold cycles) were calculated from triplicates and used for calculations of expression ratios according to (Pfaffl 2001) with primer-specific efficiencies. The Ct values for an amplicon of the SSUrRNA with primer pair SSU-f/SSU-r were used as a reference for normalization. For each primer pair, real-time experiments were carried out at least twice with biologically independent samples, and the significance of differential expression was verified using REST (Pfaffl et al. 2002).

Microscopy

A Zeiss Axiophot microscope was used for light microscopy. Pictures were captured with an AxioCam camera. Recorded images were edited using Adobe Photoshop 6.0™.

Results

Generation and phenotype analysis of the *S. macrospora* Δ *Smta-1* mutant strain

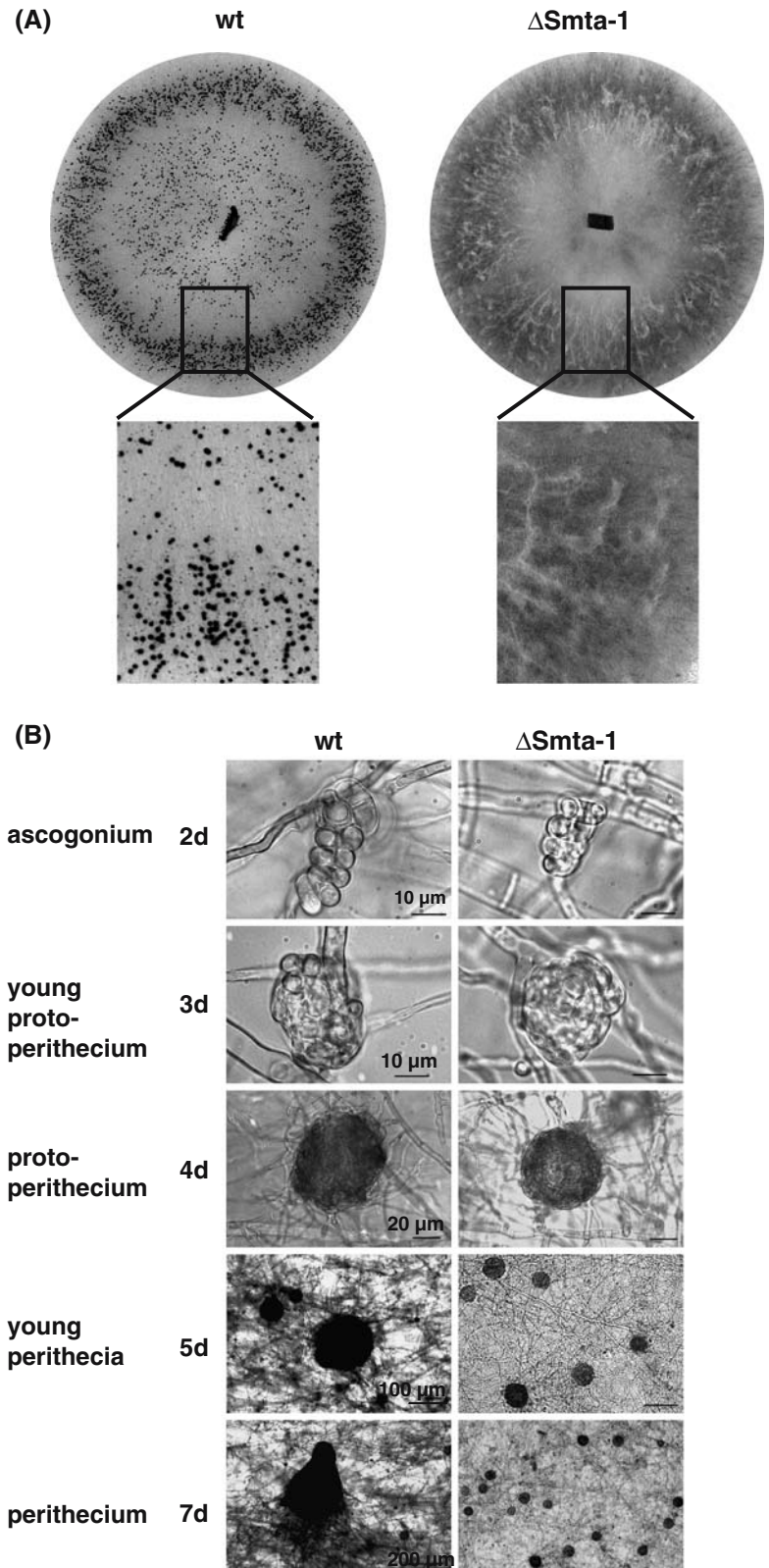
The *S. macrospora* mating-type gene *Smta-1* is predicted to encode HMG domain DNA binding protein of 288 amino acids (Pöggeler et al. 1997). To determine the role of the putative transcription factor SMTa-1 during vegetative and sexual development of the homothallic ascomycete *S. macrospora*, we generated a Δ *Smta-1* mutant strain. The knockout strain was generated as described in [Materials and methods](#) and was confirmed by Southern blot and PCR analysis (Fig. 1). To be sure that the deletion strain bears no traces of wild-type nuclei, a primary transformant (T2) carrying the expected deletion was crossed with the *S. macrospora* spore-color mutant *fus1-1* that produces brown ascospores. From this cross, we selected the hygromycin-resistant homokaryotic Δ *Smta-1* single spore isolate S59403.

Mutant strain S59403 was examined for defects in vegetative and sexual development. No irregularities in vegetative growth and mycelial morphology were seen when compared to *S. macrospora* wild type. The development of sexual reproductive structures was analyzed on corn-meal medium. Although the mutant strain showed the formation of ascogonia and protoperithecia, it was unable to perform the transition to mature

fruiting bodies (Fig. 2). When grown on fructification medium, Δ Smta-1 produced 240 protoperithecia/cm², similar to the wild-type strain (231 protoperithecia/cm²). The mean diameter of protoperithecia in mutant and

wild type is 55 and 46 μ m, respectively. However, fruiting bodies and ascospores that are formed in the wt (39 perithecia/cm²) were never observed in the Δ Smta-1 mutant even after extended incubation time. Thus, the

Fig. 2 Sexual developmental stages of the wild type (wt) and Δ Smta-1 mutant strain. **a** Strains of *S. macrospora* were grown on fructification medium for 9 days. Note the complete absence of perithecia in the mutant strain when compared with the wt strain. **b** Differential interference contrast microscopy identifies ascogonia (wt, Δ Smta-1), protoperithecia (wt, Δ Smta-1), young perithecia (only wt strain) and perithecia (only wt strain). Strains were grown on fructification medium and examined after growth at 25°C for days as indicated



mutant is completely sterile and resembles phenotypically previously described pro mutants which also do not produce any perithecia (Masloff et al. 1999; Nowrousian et al. 2005; Pöggeler and Kück 2004).

Conventional genetic analysis of more than ten ordered tetrads from a cross of the Δ Smta-1 mutant with the *fus1-1* mutant revealed a Mendelian 1:1 segregation of *fus* and Δ Smta-1 mutant phenotype. The Δ Smta-1 mutant phenotype was always linked with the hygromycin resistance, thus indicating that deletion of the *Smta-1* gene is responsible for the mutant phenotype. To further demonstrate that the phenotype was due to mutation of *Smta-1*, we complemented the mutant phenotype of Δ Smta-1 by transformation with the wild-type *Smta-1* gene. Complemented mutants were able to produce perithecia and ascospores (data not shown).

Transcriptional expression of pheromone and pheromone receptor genes

It was shown previously that the pheromone precursor genes in the heterothallic filamentous ascomycete *N. crassa* are expressed in a mating type-specific manner. The homologue of the *ppg2* gene, *mfa-1*, is expressed in *mat a* strains, whereas *cgg-4*, a homologue of the *S. macrospora ppg1* gene, is expressed in *mat A* strains (Bobrowicz et al. 2002). To examine the effect of the HMG-domain protein SMTa-1 on the expression of the pheromone precursor genes in a homothallic ascomycete, we compared mRNA expression of *ppg1* and *ppg2* in the wild type versus the Δ Smta-1 strain by means of quantitative RT-PCR experiments.

Real-time PCR experiments were performed three times with RNA prepared from independently grown mycelia. In all experiments, *ppg2* transcript levels were strongly down-regulated in the Δ Smta-1 strain compared to the wild type (Fig. 3). On average, mRNA amounts in the mutant strain were reduced about 500-fold. These results indicate that the mating type protein SMTa-1 acts as a positive regulator on the expression of the pheromone precursor gene *ppg2*. Unlike *ppg2*, transcript levels of *ppg1*, *pre1* and *pre2* varied across RT-PCR experiments, which were up-regulated in some experiments and down-regulated in some. Mean expression ratios for three experiments are given in Fig. 3, but significance analysis with REST (Pfaffl et al. 2002) indicated that none of the three genes is differentially regulated in Δ Smta-1 compared to wild type. Thus, no clear effect of the deletion of *Smta-1* on *ppg1*, *pre1* and *pre2* expression can be seen; if anything, the genes seem to be deregulated in the absence of *Smta-1*.

Microarray analysis of the Δ Smta-1 mutant

In a previous study, we have found that cross-species microarray hybridizations with *S. macrospora* targets on *N. crassa* cDNA microarrays can be used to identify

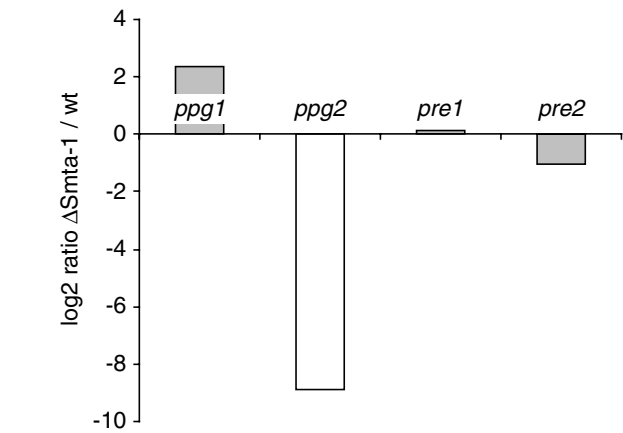


Fig. 3 Quantitative real-time PCR analysis of *ppg1*, *ppg2*, *pre1*, and *pre2* in Δ Smta-1 and the wild type. Ratios are given as logarithmic values (base 2) of means of three independent experiments, means were calculated with REST. Only *ppg2* (white bar) is differentially expressed (*P*-value 0.001, determined with REST)

developmentally regulated genes in *S. macrospora* (Nowrousian et al. 2005). We, therefore, performed cross-species microarray hybridizations with targets derived from the *S. macrospora* wild type and the Δ Smta-1 mutant on *N. crassa* cDNA arrays to identify genes that are differentially regulated in the Δ Smta-1 strain. Two hybridization experiments were performed with dye-switching in the second experiment as described previously (Nowrousian et al. 2005).

Of the 2,880 cDNA clones on the arrays, 1,063 gave hybridization signals significantly above background in all hybridizations. For these 1,063 clones, the ratio of mutant- versus wild-type expression could be determined in both independent experiments, and only these clones were used for further analysis. Among the spots that gave signals in all hybridizations, we identified 107 genes (10%) that were up- or down-regulated more than twofold in the Δ Smta-1 compared to the wild type. Of these, 74 genes were down-regulated, and 33 were up-regulated in the mutant strain (see Tables S1 and S2 in the supplementary material).

Among the genes that are down-regulated in Δ Smta-1 is *SMU4533*, the homologue of *NCU04533.2*. This gene had already been identified as down-regulated in other developmental mutants of *S. macrospora* (Nowrousian et al. 2005). Transcriptional expression of *SMU4533* in the Δ Smta-1 strain was verified by Northern blot analysis and was found to be strongly reduced (data not shown), thereby confirming that the cross-species microarray data correctly reflect expression patterns as was found before (Nowrousian et al. 2005).

In a previous analysis, we had already identified genes that are differentially regulated in the three developmental mutants *pro1*, *pro11*, and *pro22* using cross-species microarray hybridizations (Nowrousian et al. 2005). These mutants only produce protoperithecia, and thus phenotypically resemble the Δ Smta-1 strain. Therefore, we analyzed whether any gene is regulated in

a similar manner in the *pro* mutants and the Δ *Smta-1* mutant. A combined analysis of regulated genes revealed a total of 176 genes that gave hybridization signals with all four mutant strains as well as the wild type and were regulated differentially in at least one of the mutant strains. Of these, 56 genes were down-regulated and 24 genes up-regulated only in the Δ *Smta-1* mutant strain, whereas 33 genes were up-regulated and 36 genes down-regulated in one or more of the *pro* mutants, but not in the Δ *Smta-1* mutant (Table 2 and Table S1–S4 in the supplementary material). However, nine genes were down-regulated and one was up-regulated in all four mutants strains (Table 3). Thus, it seems that there is only a limited overlap among the genes regulated by *Smta-1* and the *pro* genes, respectively (Table 2). This is also reflected in the fact that the pheromone precursor genes *ppg1* and *ppg2* are up-regulated in all three *pro* mutants (Nowrousian et al. 2005), whereas *ppg2* is down-regulated in the Δ *Smta-1* strain (Fig. 3).

The EST sequences corresponding to the genes regulated differentially in Δ *Smta-1* were compared to the *Neurospora* genome database (<http://www.broad.mit.edu/annotation/fungi/neurospora/>) and to GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) using BLASTX (Altschul et al. 1997). Seventy-five percent of the regulated genes have homologues with known or putative function and were sorted into 10 putative functional categories (Fig. 4). Among these genes are a number of genes that are involved in differentiation processes in other fungi or have homology to signal transduction

Table 2 Genes that are regulated differently in mutants Δ *Smta-1*, *pro1*, *pro11* or *pro22*. Genes that are down-regulated are shaded in dark gray, up-regulated genes in light gray

Δ <i>Smta-1</i>	<i>pro1</i>	<i>pro11</i>	<i>pro22</i>	no. of genes
				9
				1
				2
				1
				2
				2
				1
				56
				2
				7
				1
				16
				3
				4
				3
				1
				1
				7
				24
				1
				2
				2
				12
				16

components. Examples are a homologue to a putative G-protein coupled receptor (NCU04931.2), the G protein γ subunit GNG-1 (NCU00041.2), a putative rho1 GDP-GTP exchange protein (NCU02131.2), an adenyl cyclase (NCU08377.2), and two serine threonine kinases (NCU06179.2; NCU00685.2). The *gng-1* and the adenyl cyclase encoding gene *cr-1* (NCU08377.2) are up-regulated, whereas all other genes are down-regulated in the Δ *Smta-1* mutant. Implications of the findings are discussed below.

Discussion

The *S. macrospora* Δ *Smta-1* mutant strain is unable to form fertile fruiting-bodies

In heterothallic filamentous ascomycetes, two important steps in sexual reproduction are regulated by the mating-type encoded transcription factors: (1) the initial fertilization event mediated by chemo-attraction between reproductive structures of two compatible partners and (2) before karyogamy, the paired migration of nuclei of opposite mating types into the ascogenous hyphae (Coppin et al. 1997; Shiu and Glass 2000). Homothallic fungi have no genetically defined mating type and typically carry one mating-type locus that incorporates homologues from genes of both mating types of related heterothallic species (Pöggeler et al. 1997; Yun et al. 1999). In contrast to the heterothallic ascomycetes, fruiting body development is an apandrous process in the homothallic *S. macrospora* and lacks the cooperative interaction of two opposite mating-type strains. Sexual development starts with the formation of female gametangia called ascogonia. The ascogenous cells are enveloped by sterile hyphae to form protoperithecia as fruiting body precursors; conidiospores, spermatia, and trichogynes are lacking. After autogamous fertilization (i.e. pairwise fusion of nuclei present within the ascogonium, without cell fusion having taken place), the protoperithecia differentiate inner ascus initials, embedded in sterile paraphyses, and an outer-pigmented peridial tissue. Meiosis and a postmeiotic division lead to eight meiotically derived ascospores in each ascus (Esser 1982; Esser and Straub 1958). Thus, although the fertilization process does not involve two genetically distinct nuclei, the homothallic *S. macrospora* must ensure that karyogamy occurs exactly between two nuclei. Therefore, it has been proposed that individual nuclei of homothallic fungi could be functionally heterothallic in order to allow nucleus recognition (Coppin et al. 1997; Glass et al. 1990b). This might be achieved through differential expression of mating-type genes (Pöggeler and Kück 2000).

Furthermore, it was not clear whether *mata* genes are essential for sexual reproduction in homothallic members of the family Sordariaceae, as some homothallic *Neurospora* species carry only *matA* specific genes but no *mata* genes (Glass et al. 1990b; Glass and Smith 1994)

Table 3 Genes that are regulated similarly in Δ Smta-1 and three pro mutants

<i>N. crassa</i> acc. no.	<i>N. crassa</i> ORF	Closest homologue with known/putative function	Homology	Log2 ratio mutant/wt			
				Δ Smta-1	pro1	pro11	pro22
ref XP_331026.1	NCU02250.2	sp Q03672	Mitochondrial ATP synthase protein 9 (<i>Podospora anserina</i>)	2.16	2.15	1.70	2.24
ref XP_331037.1	NCU02261.2	gb AAW42150.1	Putative NEDD8 activating enzyme (<i>Cryptococcus neoformans</i>)	-3.52	-3.30	-3.20	-3.05
ref XP_331731.1	NCU02532.2	gb AAC47135.1	Calcium-dependent cell adhesion molecule (<i>Dictyostelium discoideum</i>)	-1.53	-2.10	-2.21	-3.09
ref XP_323886.1	NCU04533.2	gb AAL12251.1	Mold-specific MS8 protein (<i>Ajellomyces capsulatus</i>)	-3.64	-4.27	-3.12	-2.98
ref XP_324285.1	NCU04928.2	emb CAE55153.1	Putative G protein-coupled receptor (<i>Botryotinia fuckeliana</i>)	-3.31	-2.22	-3.07	-3.11
ref XP_324288.1	NCU04931.2	emb CAA16826.1	sepl, forkhead transcription factor homologue (<i>Schizosaccharomyces pombe</i>)	-3.24	-2.90	-3.52	-3.29
ref XP_326028.1	NCU06173.2	gb AAT40415.1	b-ZIP transcription factor IDI-4 (<i>Podospora anserina</i>)	-1.98	-1.21	-2.17	-1.50
ref XP_328761.1	NCU08055.2	gb AAK07185.1	Glucose-6-phosphate 1-dehydrogenase trihydroxynaphthalene reductase (<i>Ophiostoma floccosum</i>)	-2.18	-1.09	-1.54	-1.60
ref XP_331503.1	NCU09111.2			-1.85	-1.02	-1.77	-1.33
ref XP_332082.1	NCU09390.2			-2.32	-1.68	-2.01	-2.19

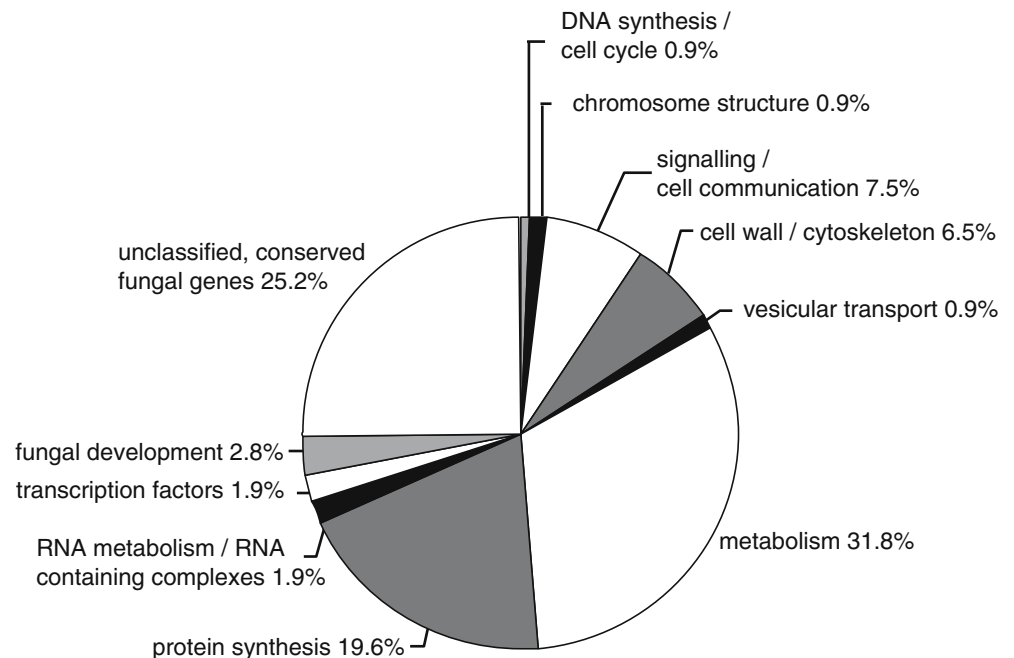
and *matA* strains of the heterothallic *Sordaria brevicollis* have been shown to produce fruiting bodies with ascospores under certain cultural conditions (Robertson et al. 1998). This study demonstrates that the *Smta-1* gene is required for fruiting body development and sexual reproduction in the homothallic *S. macrospora*. Deletion of *Smta-1* encoding a putative HMG-domain transcription factor converts the self-fertile *S. macrospora* to a self-sterile fungus, no longer able to produce fruiting bodies and ascospores. Similarly, it has been previously demonstrated that deletion of the HMG domain protein encoding *MAT1-2* gene, a putative orthologue of the *S. macrospora Smta-1*, in the homothallic Nectriaceae *Gibberella zeae* resulted in a self-sterile

strain. In *G. zeae*, the *MAT1-2* deletion strain was still able to outcross with a homothallic tester strain (Lee et al. 2003). Likewise, the *S. macrospora* Δ Smta-1 strain displayed a 1:1 segregation of the *hph* marker gene when outcrossed with the *fus1-1* mutant, thus indicating that the Δ Smta-1 strain has retained the ability to be sexual.

Expression of the pheromone precursor gene *ppg2* is controlled by the mating type protein SMTa-1

In ascomycetes, two types of pheromone precursor genes are present in the same genome. For the yeast *S. cerevisiae*, it has been shown that expression of pheromone

Fig. 4 Functional classification of differentially regulated genes. In microarray experiments with targets prepared from wild type and the *S. macrospora* Δ Smta-1 mutant, we found 74 to be down- and 33 to be up-regulated in the mutant strain. BLASTX analysis of the 107 regulated genes yielded 80 genes (75%) with a homologue with known or putative function. Twenty-seven genes encode unknown but conserved fungal proteins



genes is directly regulated by transcription factors encoded by the mating-type locus (Herskowitz 1989).

While in heterothallic filamentous ascomycetes such as *N. crassa*, *Magnaporthe grisea*, and *Podospora anserina*, pheromone genes are expressed in a mating-type dependent manner, both genes are expressed in the homothallic *S. macrospora* having *a*- and *A*-specific transcription factors in its mating-type locus (Bobrowicz et al. 2002; Coppin et al. 2005; Pöggeler et al. 1997, 2000; Shen et al. 1999). It was demonstrated for the heterothallic *P. anserina* that mating-type genes activate their specific pheromone gene and repress the complementary pheromone gene (Coppin et al. 2005).

In our study, the Δ Smta-1 mutant that lacks the *a*-specific HMG transcription factor SMTa-1 displayed a 500-fold reduction of lipopeptide *ppg2* gene expression; thus indicating that SMTa-1 has a direct or indirect impact on the activation of *ppg2* gene expression. Similarly, it was demonstrated for the heterothallic *N. crassa* that the *a*-specific pheromone gene *mfa-1* was not expressed in a *mat a^{ml}* mutant strain that has a frameshift mutation in the *mat a1* coding region (Bobrowicz et al. 2002; Staben and Yanofsky 1990). Previously, it was shown that the *N. crassa* MATa-1 polypeptide produced in *E. coli* binds to specific DNA sequences whose core is CTTTG or CTTCG and that DNA binding is a function of the HMG domain (Philly and Staben 1994). The CTTTG motif corresponds to the pheromone response element recognized by the PRF1 HMG transcription factor in the basidiomycete *Ustilago maydis* (Hartmann et al. 1996). The *S. macrospora* SMTa-1 protein shows 86.0% identity with the corresponding Mata-1 protein of *N. crassa*, and the 80-aa HMG domain of both proteins display even 96.0% of identity, suggesting that the cellular functions and the specific DNA-binding recognition sites for both proteins are highly similar, possibly identical. Analysis of the 5' non-coding region of the *S. macrospora ppg2* gene revealed the presence of the above-mentioned HMG-domain protein binding sequences: two CTTTG and four CTTCG sequences. These sequences, similar to the *N. crassa* binding sites, might thus be binding sites for the SMTa-1 protein, which might then directly activate transcription of *ppg2*. However, the action of SMTa1 on *ppg2* transcription might also be an indirect activation of a transcriptional activator that again might bind the 5' noncoding sequence of *ppg2*.

In contrast, no clear effect on the expression of the complementary pheromone gene *ppg1* gene has been observed in the *S. macrospora* Δ Smta-1 strain. Similarly, the deletion of *Smta-1* had no clear effect on the transcriptional expression of the pheromone receptor genes *pre1* and *pre2*. This result does not come as a surprise, since transcription of pheromone receptor genes was previously shown to be mating-type independent in the heterothallic *N. crassa* (Pöggeler and Kück 2001). However, in our study we only analyzed transcriptional expression of *ppg1*, *pre1* and *pre2*; therefore, a post-transcriptional effect of SMTa-1 on the expression of

these genes cannot be ruled out. In *P. anserina*, it was recently shown that the orthologue of SMTa-1, FPR1, not only activates the expression of the *mat*- specific pheromone gene but also represses the expression of the complementary *mat*⁺ specific pheromone gene post-transcriptionally (Coppin et al. 2005).

The mating type protein SMTa-1 affects the expression of several genes including a common set of developmental genes deregulated in sterile pro mutants

Since a Δ ppg2 pheromone knockout mutant is still able to form fruiting-bodies and ascospores, the 500-fold down-regulation of *ppg2* gene expression cannot be responsible for the sterile phenotype of Δ Smta-1 (Mayrhofer et al. 2006). To examine the role of SMTa-1 in the regulation of other genes that might be target genes of SMTa-1, we performed a cross-species microarray analysis. Following signal intensity quantification and normalization, 107 genes showed significant alterations in mRNA abundance in the Δ Smta-1 mutant. Among these, a BLAST search identified 80 genes with known or putative functions (Fig. 4).

The analysis of three other independent mutants affected in fruiting body formation showed that only a limited set of genes are deregulated in a similar way. Mutants, *pro1*, *pro11* and *pro22*, carrying mutations in different genes have a developmental block just after protoperithecia formation and, therefore, resemble the phenotype of Δ Smta-1. The *pro1* gene encodes a zinc finger transcription factor, *pro11* encodes a WD40 repeat protein and *pro22* a putative membrane protein (Masloff et al. 1999; Nowrousian et al. 2005; Pöggeler and Kück 2004). Only ten genes were regulated similarly in all mutants (Table 2). Thus, this set of deregulated genes seems to be fruiting body development specific rather than mating-type specific regulated. Among the genes down-regulated in all mutants is *NCU04931.2* (Table 3 and Tables S1 and S2 of the supplementary material). The protein encoded by *NCU04931.2* is predicted to have seven transmembrane spanning domains (<http://www.enzim.hu/hmmtop/>) and is a putative homologue of the recently described seven-helix transmembrane protein BTP1 of the asexual plant pathogen *Botrytis cinerea*. In *B. cinerea*, this putative G-protein coupled receptor was suggested to play a role in mediation of plant signals and pathogenicity. However, targeted gene replacement of the *B. cinerea bpt1* did not result in a phenotype affecting either pathogenicity or sensitivity to chemical stress (Schulze Gronover et al. 2005). Since *B. cinerea* reproduces only asexually, influences of this receptor on the development of fruiting bodies have not been described yet. The putative receptor may play a role in targeting signals relevant for sexual development in *S. macrospora*. Down-regulated expression in all mutants was also found for two genes encoding putative transcription factors. A putative bZIP transcription factor (*NCU08055.2*) and a putative fork

head domain transcription factor (*NCU06173.2*) showed a fourfold to fivefold decrease in mRNA level in Δ Smta-1 and are also down-regulated in the pro mutants (Table 3, supplementary material). *NCU08055.2* is a putative homologue of IDI-4, a bZIP transcription factor inducing autophagy and cell death in *P. anserina* when overexpressed (Dementhon et al. 2004). The second transcription factor gene down-regulated in Δ Smta-1 and the pro mutants, *NCU06173.2*, is a homologue of the *S. pombe* forkhead transcription factor Sep1. The *S. pombe* Sep1 protein regulates mitotic gene transcription and is involved in the cell separation at the end of mitosis. (Buck et al. 2004; Ribar et al. 1999). Another gene that is down-regulated in all mutants is a homologue of *NCU02532.2* encoding a putative NEDD8 activating enzyme (Liakopoulos et al. 1998). NEDD8 is an ubiquitin-like molecule that covalently ligates to members of the cullin-family proteins through an enzymatic cascade analogous to ubiquitylation. Previously, it has been demonstrated that the COP9 signalosome (CSN) which interacts with cullin-containing proteins is a regulator of fruiting body development in *Aspergillus nidulans* (Busch et al. 2003). Among the genes down-regulated in all mutants are, in addition, genes involved in cell wall biogenesis and structure (*NCU04533.2* and the trihydroxynaphthalene reductase homologue *NCU09390.2*, Table 3). The homologue of *NCU04533.2* was demonstrated to be highly expressed during sexual development of *S. macrospora* (Nowrousian, unpublished), while trihydroxynaphthalene reductase is most probably necessary for ascospore and perithecial cell wall pigmentation (Wang and Breuil 2002).

Of the 107 genes that were regulated differentially in the Δ Smta-1 mutant, 56 and 24 genes were only down- and up-regulated, respectively, in the Δ Smta-1 mutant and not in the pro mutants (Table 2, see also Tables S1 and S2 supplementary material). Thus, the regulation of these genes seems to be *Smta-1*-specific rather than fruiting body specific. These data indicate that *Smta-1* is involved in the regulation of pathways distinct from pathways regulated by the *pro* genes. Of the 80 genes regulated differentially in Δ Smta-1 alone, several encode components of different signal transduction pathways. The gene homologous to *NCU00041.2* encodes the γ -subunit GNG-1 of a heterotrimeric G-protein. In the Δ Smta-1 mutant, the *gng-1* gene is up-regulated. Recently it was demonstrated that deletion of *gng-1* negatively influences levels of G α proteins (GNA-1, GNA-2, and GNA-3) in plasma membrane fractions isolated from various tissues of *N. crassa* and leads to a significant reduction in the amount of intracellular cyclic AMP (Krystofova and Borkovich 2005). Thus, up-regulation of *gng-1* in the Δ Smta-1 might lead to an up-regulation of genes encoding G α subunits and adenylate cyclases. Interestingly, our study revealed that indeed the transcript level of adenylate cyclase gene *cr-1* (*NCU08377.2*) was elevated in the Δ Smta-1 mutant. In *N. crassa* it has been shown previously that the adenylate cyclase together with the G α subunit GNA-1 contributes to proper

fruiting body and ascospore development (Ivey et al. 2002). Similarly, cAMP signaling might be involved in sexual development of *S. macrospora*.

Another component of a signal transduction pathway exhibiting a more than five-fold decreased mRNA level in the Δ Smta-1 mutant is the homologue to *NCU02131.2*. The *N. crassa* gene encodes a putative homologue of the *S. pombe* guanine nucleotide exchange factor Rgfp3. In *S. pombe*, Rgfp3 regulates the activity of the small GTPase Rho1p which is involved in β -1,3-glucan biosynthesis and cell integrity during septation (Tajadura et al. 2004). In this context, it is worth noting that in Δ Smta-1 the *NCU04431.2* homologue encoding a putative endo-1,3- β -glucanase is one of the most strongly down-regulated genes. In addition to this gene, the expression of further six genes involved in cell wall biosynthesis is deregulated in Δ Smta-1.

The genes affected by SMTa-1 are numerous and include the pheromone gene *ppg2* and several genes for components of signaling cascades. Deletion of *Smta-1* results in sterility of *S. macrospora* and thus, similar to other sterile mutants, genes up-regulated during fruiting body development are down-regulated in the Δ Smta-1 mutant. Taken together, the mating-type proteins of homothallic filamentous ascomycetes seem to control sexual reproduction by regulating a variety of essential cellular processes. Further studies are necessary to elucidate the molecular mechanism underlying this complex regulatory network.

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