Molecular Determinants of Sporulation in *Ashbya gossypii*

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ABSTRACT Regulation of development and entry into sporulation is critical for fungi to ensure survival of unfavorable environmental conditions. Here we present an analysis of gene sets regulating sporulation in the homothallic ascomycete *Ashbya gossypii*. Deletion of components of the conserved pheromone/starvation MAP kinase cascades, e.g., *STE11* and *STE7*, results in increased sporulation. In *kar3* mutants sporulation is severely reduced, while deletion of *KAR4* as well as of homologs of central *Saccharomyces cerevisiae* regulators of sporulation, *IME1*, *IME2*, *IME4*, and *NDT80*, abolishes sporulation in *A. gossypii*. Comparison of RNAseq transcript profiles of sporulation-deficient mutants identified a set of 67 down-regulated genes, most of which were up-regulated in the oversporulating *ste12* mutant. One of these differentially expressed genes is an endoglucanase encoded by *ENG2*. We found that Eng2p promotes hyphal fragmentation as part of the developmental program of sporulation, which generates single-celled sporangia. Sporulation-deficient strains are arrested in their development but form sporangia. Supply of new nutrients enabled sporangia to return to hyphal growth, indicating that these cells are not locked in meiosis. Double-strand break (DSB) formation by Spo11 is apparently not required for sporulation; however, the absence of *DMC1*, which repairs DSBs in *S. cerevisiae*, results in very poor sporulation in *A. gossypii*. We present a comprehensive analysis of the gene repertoire governing sporulation in *A. gossypii* and suggest an altered regulation of *IME1* expression compared to *S. cerevisiae*.

A *SHBYA gossypii* is a riboflavin/vitamin B₂ overproducing filamentous ascomycete grouped within the *Saccharomycetaceae* (Kurtzman and Robnett 2003). *A. gossypii* belongs to the pre-whole genome duplication fungi but shares homologs of 95% of its genes with *Saccharomyces cerevisiae* (Deng *et al.* 2007). *A. gossypii* and its relative *Eremothecium cymbalariae* can complete their life cycle starting from a single spore that forms a sporulating mycelium and are thus homothallic (Wendland and Walther 2011). Sporulation in *A. gossypii* occurs at the end of its growth phase, when hyphae develop sporangia derived from septate compartments. Sporangia separate from each other by fragmentation of the hyphae at septal sites and spores are set free by lysis of the sporangia. *A. gossypii* spores are uninucleate and contain a haploid genome (Wendland and Walther 2005).

In *S. cerevisiae* mating pheromones induce signaling via a conserved MAP kinase cascade that leads to the activation of the *Ste12* transcription factor. *Ste12* binds to the pheromone

response element of target genes and induces their expression, which induces events leading to cell fusion and karyogamy (Bardwell 2005). In *A. gossypii* mating is apparently not required for sporulation as mutant strains deleted for both *STE2* and *STE3* pheromone receptor genes can still sporulate. On the contrary, deletion of the *AgSTE12* homolog resulted in an oversporulation phenotype (Wendland *et al.* 2011).

Meiosis and sporulation do not occur in haploid yeast cells, which is due to at least two regulatory mechanisms. First, *RME1* acts as a repressor of meiosis by inhibiting expression of *IME1*, the inducer of meiosis. To this end, *Rme1* activates the transcription of a long noncoding RNA, *IRT1*, in the *IME1* promoter (Van Werven *et al.* 2012). Second, expression of the meiotic regulatory protein *IME4* in haploid cells is abolished by an *IME4* antisense transcript, *RME2* (Hongay *et al.* 2006). Both repression mechanisms are alleviated in *MATa/α* diploid cells. Here the *a1/α2* heterodimer represses the expression of *RME1* and the expression of the antisense transcript at the *IME4* gene (Mitchell and Herskowitz 1986; Hongay *et al.* 2006). To maintain this intricate mechanism, diploid cells depend upon Dig1 and Dig2 regulators as deletion of both genes results in activation of *Ste12* and enables diploid cells to mate preferentially as *a* cells (Gelli

2002). Furthermore, sporulation in diploid cells is controlled by environmental cues and only proceeds upon nitrogen starvation and the presence of a nonfermentable carbon source (Kassir *et al.* 2003). In *S. cerevisiae*, nitrogen starvation is signaled via a MAPK cascade that uses elements of the pheromone response and activates *Tec1* (Figure 1). In *S. cerevisiae* these environmental signals are integrated at the *IME1* promoter. Onset of *IME1* expression results in the activation of the sporulation program, which requires the key regulatory genes *IME2*, *IME4*, *UME6*, and *NDT80* (Neiman 2011).

The developmental program resulting in sporulation in *A. gossypii* has not been analyzed so far. Based on the genome sequence, it is apparent that restrictions such as in haploid *S. cerevisiae* cells do not apply: (i) the *A. gossypii* genome does not encode an *RME1* gene and (ii) the standard laboratory strain harbors only *MATa* loci encoding *a1* and *a2* proteins and thus does not produce an *a1/a2* heterodimer (Dietrich *et al.* 2004; Wendland and Walther 2005). Currently, it is unknown, whether karyogamy and meiosis occur in *A. gossypii*. Based on our previous findings that *A. gossypii* homologs of the pheromone receptors *STE2* and *STE3* can be deleted without inhibiting sporulation, we hypothesized that *A. gossypii* evolved a specialized homothallism based on homokaryotic haploid fruiting (Wendland *et al.* 2011).

Here we describe the analysis of other components of the pheromone response pathway. We found that, in contrast to *S. cerevisiae*, *GPA1* deletion is not lethal in *A. gossypii*. We also identify that *STE11* and *STE7* mutants produce spores more abundantly than the wild type. Signaling via the pheromone/starvation MAPK-cascades is important for sporulation in *A. gossypii*: *ste12* and *tec1* strains oversporulate (Wendland *et al.* 2011), while deletion of *DIG1*, an inhibitor of both *Ste12* and *Tec1* in *S. cerevisiae*, abolishes sporulation. Characterization of *A. gossypii* homologs of *S. cerevisiae* sporulation regulators resulted in the identification of sporulation-deficient strains, including *ime1*, *ime2*, *kar4*, and *ndt80*. Transcription profiles of these strains identified down-regulated genes that are up-regulated in the oversporulating *ste12* mutant. One of these differentially expressed genes, encoded by *ENG2*, promotes hyphal fragmentation.

Materials and Methods

Strains and media

A. gossypii strains were grown in yeast extract-peptone-dextrose [Ashbya Full Medium (AFM); 1% yeast extract, 1% peptone, 2% dextrose] and G418/geneticin (200 μ g/ml) or clonNAT (100 μ g/ml) was added for selection of antibiotic-resistant transformants. For sporulation, an overnight culture of *A. gossypii* was further incubated in minimal medium [1.7 g/liter Yeast Nitrogen Base (YNB) w/o ammonium sulfate and w/o amino acids, 0.69 g/liter Complete Synthetic Medium (CSM), 20 g/liter glucose, 2 g/liter asparagine, and 1 g/liter myoinositol] for up to 5 days. The *A. gossypii* deletion mutants generated in this study are listed in Table 1. For each desired

Figure 1 Diagram of the MAP kinase pathways that govern the mating pheromone response and starvation/filamentous growth response in *Saccharomyces cerevisiae*. MEKK, MEK kinase; MEK, MAP/ERK kinase; MAPK, mitogen-activated kinase.

genetic manipulation, two independent transformants were generated. Strains were further manipulated by transforming them with freely replicating plasmids which are listed in Table 2. *S. cerevisiae* BY4741 (*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was used for *in vivo* recombination. *A. gossypii* and *S. cerevisiae* strains were grown at 30°. *Escherichia coli* strain DH5α was used for plasmid propagation and grown at 37°.

Transformation of *A. gossypii* and *S. cerevisiae*

A. gossypii strains were transformed by electroporation as described previously (Wendland *et al.* 2000). Yeast cells were transformed by the lithium-acetate procedure (Gietz and Schiestl 2007). PCR-based cassettes amplified from pFA-GEN3 using gene specific S1- and S2 primers were used as transforming DNA for *A. gossypii* as described (Wendland *et al.* 2000). Marker exchange in pAG plasmids was achieved by yeast *in vivo* recombination to exchange the *URA3* marker for the *NAT4* marker. To this end, *NAT4* cassettes were amplified from pRS-NAT4 with primers #6062 and #6063. All primers were purchased from Integrated DNA Technologies (IDT, Leuven, Belgium). Primer sequences will be made available upon request. Diagnostic PCR was used for verification of correct integration of a disruption cassette and concomitant absence of the target gene in homokaryotic deletion strains.

Plasmid constructs

Episomal plasmids were generated for complementation experiments with the *A. gossypii* mutant strains *ime1*, *ime2*, *ime4*, *kar4*, *ndt80*, and *ume6*. For *IME1* and *KAR4*, the full-length genes were excised from the pAG-clones #13310 and #7087, respectively, and cloned into pRS418 using *SacI/XhoI* restriction sites for *IME1* and *XhoI/XbaI* restriction sites for

Table 1 *A. gossypii* deletion strains generated in this study

Strain	Genotype
ALK1-1	<i>leu2, spo1::GEN3/SPO1</i>
ALK1-10	<i>leu2, spo1::GEN3</i>
ALK10-1	<i>ste7::GEN3 leu2/STE7</i>
ALK10-10	<i>ste7::GEN3 leu2</i>
ALK12-1	<i>ste11::GEN3 leu2/STE11</i>
ALK12-10	<i>ste11::GEN3 leu2</i>
ALK25-1	<i>leu2, ndt80::GEN3/NDT80</i>
ALK25-10	<i>leu2, ndt80::GEN3</i>
ALK36-1	<i>leu2, eng1::GEN3/ENG1</i>
ALK36-10	<i>leu2, eng1::GEN3</i>
ALK37-1	<i>leu2, eng2::GEN3/ENG2</i>
ALK37-10	<i>leu2, eng2::GEN3</i>
ALK44-1	<i>leu2, ime1::GEN3/IME1</i>
ALK44-10	<i>leu2, ime1::GEN3</i>
ALK55-1	<i>leu2, dig1::GEN3/DIG1</i>
ALK55-10	<i>leu2, dig1::GEN3</i>
AWE78	<i>leu2, gpa1::kanMX/GPA1</i>
AWE84	<i>leu2, gpa1::kanMX</i>
AWE80	<i>leu2, ste4::kanMX/STE4</i>
AWE85	<i>leu2, ste4::kanMX</i>
AWL-16	<i>leu2, fus3::GEN3/FUS3</i>
AWL-47	<i>leu2, fus3::GEN3</i>
AWL-10	<i>leu2, ime4::GEN3/IME4</i>
AWL-41	<i>leu2, ime4::GEN3</i>
AWL-18	<i>leu2, ime2::GEN3/IME2</i>
AWL-43	<i>leu2, ime2::GEN3</i>
AWL-29	<i>leu2, ume6::GEN3/UME6</i>
AWL-67	<i>leu2, ume6::GEN3</i>
AWL-71	<i>leu2, kar1::GEN3/KAR1</i>
AWL-73	<i>leu2, kar2::GEN3/KAR2</i>
AWL-75	<i>leu2, kar3::GEN3/KAR3</i>
AWL-76	<i>leu2, kar3::GEN3</i>
AWL-79	<i>leu2, kar4::GEN3/KAR4</i>
AWL-80	<i>leu2, kar4::GEN3</i>
AWL-85	<i>leu2, spo11::GEN3/SPO11</i>
AWL-86	<i>leu2, spo11::GEN3</i>
AWL-91	<i>leu2, dmc1::GEN3/DMC1</i>
AWL-92	<i>leu2, dmc1::GEN3</i>
AWL-99	<i>leu2, kar5::GEN3/KAR5</i>
AWL-100	<i>leu2, kar5::GEN3</i>
AWL-83	<i>leu2, kar8::GEN3/KAR8</i>
AWL-103	<i>leu2, kar8::GEN3</i>
AWL-138	<i>leu2, spo14::GEN3/SPO14</i>
AWL-139	<i>leu2, spo14::GEN3</i>

All strains are derivatives of *AgLeu2*.

KAR4. *pRS-UME6* was generated from pAG11954 by replacing the *URA3* marker with the *NAT4* marker using *in vivo* recombination in *S. cerevisiae*. *NAT4* consists of the clonNAT resistance ORF under control of *ScLEU2* promoter and terminator regions derived from pRS415. The *IME2* and *IME4* genes were amplified using the primers #5087/#5088 and #5089/#5090, respectively, and cloned into pRS418 using *XhoI/XbaI* restriction sites introduced with the amplification primers. The *NDT80* gene was amplified using primers #5091/#5092, cloned into pGEM, and recloned into pRS418 using the *SpeI* restriction site provided in pGEM and the *EcoRV* restriction site introduced with the amplification primer #5091.

The disruption cassette for *GPA1* was obtained by fusion PCR. To this end 5'- and 3'-flanking homology regions were

Table 2 Plasmids generated in this study

Plasmid	Description
C218	pRS418
C407	pSK- <i>gpa1::kanMX</i>
C414	pSK- <i>ste4::kanMX</i>
C837	pRS418- <i>AgIME1</i>
C734	pRS418- <i>AgIME2</i>
C736	pRS418- <i>AgIME4</i>
C839	pRS418- <i>AgTEF1p-AgIME4</i>
C780	pRS418- <i>AgKAR4</i>
C710	pRS418- <i>AgNDT80</i>
C764	pRS- <i>AgUME6</i>

pRS plasmids harbor a clonNAT resistance marker and are freely replicating in *A. gossypii*.

amplified using primer pairs #3858/#3859 and #3860/#3861 and added in a second PCR reaction to the *kanMX4* selectable marker gene. This fusion PCR product was cloned into pBluescript-SK (+) using *EcoRI/XhoI* sites. The disruption cassette for *STE4* was obtained by amplifying a 1961-bp fragment containing *STE4* using primers #3864 and #3865. This PCR fragment was cloned as a 1902-bp *XhoI/EcoRI* fragment into pBluescript-SK (+) using internal restriction sites. An internal *EcoRV* fragment containing most of the *STE4*-ORF was replaced by *kanMX4*.

Microscopy and staining procedures

Fluorescence microscopy was done with a Zeiss AxioImager M1. Images were acquired using a Coolsnap HQ camera (Photometrics, Tucson). Metamorph 7 software was used for automated image acquisition and data analysis. For DAPI staining cells/mycelia were fixed with 70% ethanol and washed with PBS prior to the addition of 0.1 µg/ml of DAPI. Images were acquired using a DAPI filter (excitation of 350/25 nm, emission: 460/25 nm). For chitin staining, 25 µM calcofluor white (Fluorescent Brightener 28; Sigma) was added to PBS-washed mycelia followed by incubation for 10 min in the dark at room temperature. Fluorescence microscopy was done using the DAPI filter set. For visualization of vacuoles the lipophilic dye FM4-64 was used. Sporangia or mycelia were washed and resuspended in fresh AFM medium prior to the addition of 8 µM FM4-64. After an incubation of 30 min, sporangia or mycelia were washed again and incubated for an additional 30 min before microscopy (filter set used: excitation: 545/15 nm, emission: 620/30 nm). *Actin* staining was done with Alexa Fluor 488 phalloidin (filter: excitation: 470/20 nm, emission: 525/25 nm) or rhodamine phalloidin (filter: excitation: 545/15 nm, emission: 620/30 nm). The staining procedure was performed as described previously (Walther and Wendland 2004). All dyes were purchased from Life Technologies (Naerum, Denmark).

RNA-isolation-RT-PCR

Two independent homokaryotic strains were used for each experiment. Mycelia of these strains were grown for 4 days on AFM plates and were then transferred into liquid AFM medium in baffled flasks and further incubated for 16 hr at

Table 3 Comparisons between *S. cerevisiae* and *Eremothecium* proteins

Function	Protein	<i>S. cerevisiae</i> gene	<i>A. gossypii</i> gene	Sporulation
Pheromone response	Ste2	YFL026W	AFR522C	Yes ^a
	Ste3	YKL178C	AEL131C	Yes ^a
	Gpa1	YHR005C	ADR153C	Yes
	Ste4	YOR212W	ACR097W	Yes
MAP-kinase signaling	Ste11	YLR362W	ABL011C	Increased
	Ste7	YDL159W	ACR196C	Increased
	Fus3	YBL016W	AFR019W	Yes
Transcriptional regulators		YPL049C		
	Dig1/2	YDR480W	AGL258C	None
	Ste12	YHR084W	ADR304W	Increased ^a
	Tec1	YBR083W	AER177W	Increased ^a
Involved in karyogamy	Kar1	YNL188W	ABL149W	Essential gene
	Kar2	YJL034W	ACR038W	Essential gene
	Kar3	YPR141C	AGR253W	Severely reduced
	Kar4	YCL055W	AFR736C	None
	Kar5	YMR065W	ADR143W	Yes
	Kar8	YJL073W	ADR124C	Yes
Meiotic regulators	Ime1	YJR094C	AGL308W	None
	Ime2	YJL106W	AFR076W	None
	Ime4	YGL192W	AFR173W	None
	Ndt80	YHR124W	AGR347W	None
	Ume6	YDR207C	AGL099C	Severely reduced
Sporulation	Dmc1	YER179W	AGR101C	Severely reduced
	Spo1	YNL012W	AAL027W	Reduced
	Spo11	YHL022C	ADR025W	Yes
	Spo14	YKR031C	AFR071W	Severely reduced

Function is according to *Saccharomyces cerevisiae*.

^a Sporulation phenotypes of these mutants have been reported previously (Wendland *et al.* 2011).

30°. Equal wet weights of each culture were washed and transferred to 5 ml minimal CSM medium containing 1g/liter myoinositol. The cultures were grown in baffled flasks for 16 hr at 30°. Mycelia were harvested and RNA was isolated using the RiboPure-Yeast kit (Life Technologies). RNA was treated with DNase for 1 hr and 1 µl RNA (700 ng, measured using a Nanodrop 2000 device) was used for cDNA synthesis (Reverse Transcriptase kit, Sigma; STR1-1KT). Synthesis of cDNA using the intron-containing *PFY1* gene served as quality control of the cDNA. This RNA was used for RNA sequencing analysis using an Illumina HiSeq 2000 platform.

RNA-seq

One lane of Illumina HiSeq 2000 was used for generating raw sequence data on total RNA. Illumina adapters were clipped from the reads, short reads were discarded, and reads were filtered to exclude rRNAs and tRNAs using RiboPicker 0.4.3. (<http://ribopicker.sourceforge.net/>). Formatted alignment files were generated by TopHat 2.0.4 (<http://tophat.cbcb.umd.edu/>) to generate datasets with read counts for each annotated gene with htseq-count. Differential expression analysis was done using edgeR (<http://www.bioconductor.org/packages/release/bioc/html/edgeR.html>), DESeq (<http://bioconductor.org/packages/release/bioc/html/DESeq.html>), and cufflinks/cuffdiff (<http://cufflinks.cbcb.umd.edu>).

The raw *P*-values from the statistical tests were adjusted for multiple testing by the Benjamini–Hochberg false discovery rate (FDR) method. Genes for which the tests had an FDR <5% (*i.e.*, 0.05) were considered to be differentially expressed. RNAseq was performed by LGC Genomics (Berlin).

Analysis of hyphal outgrowth from sporangia

Strains were incubated in liquid sporulation media for 5 days. Sporangia were diluted and plated onto rich medium. Outgrowth of sporangia was monitored over time using a Zeiss Axiovert 200M with a Coolsnap cf camera (Photometrics) and Metamorph 6 software. Images were acquired at time point 0 and return to growth (RTG) of the same sporangia was analyzed after 18 hr. The percentage of cells able to return to vegetative growth is presented as percentage regrowth. For each mutant, >100 sporangia were analyzed.

Results

Deletion analysis of components of the pheromone response pathway

Previously, we have studied several homologs of the pheromone response signal transduction cascade in *A. gossypii*. These included the candidate α -factor and α -factor pheromone genes, the pheromone receptor genes *STE2* and *STE3*, and the

Figure 2 Reduced vegetative growth in mutants of sporulation regulators. (A) Comparison of radial growth rates of the parental *leu2* strain and the *kar3* mutant at different temperatures. (B) Comparison of growth curves of strains under either 22°, 30°, or 37°. Strains were grown on full medium plates and radial growth was measured between days 3 and 7. Curves represent duplicate experiments of the independent homokaryotic mutant strains for each gene. (C) Complementation of growth defects in *ime4*, *kar4*, and *ume6* mutants. Strains were transformed with episomal plasmids bearing the wild-type gene. For complementation of the growth defect of *ime4*, overexpression of *IME4* from plasmid was required. As a control, the parental strain was transformed with an empty vector.

STE12 transcription factor homolog. A key finding was that *A. gossypii* can complete its life cycle from a single spore to a sporulating mycelium in the absence of *STE2*, *STE3*, or *STE12*. On the contrary, deletion of *STE3* produced a mild, and deletion of *STE12* a pronounced, oversporulation phenotype (Wendland *et al.* 2011). In *S. cerevisiae* the pheromone signal is transduced from *Ste3* via a G protein and a MAP kinase cascade onto *Ste12* (Figure 1). We, therefore, generated deletion mutants in *GPA1* and *STE4*, encoding the $G\alpha$ - and $G\beta$ -subunits of the pheromone-dependent heterotrimeric G protein, and in *STE11*, *STE7*, and *FUS3* encoding the MAP kinase module. All deletion mutants are viable and grow with radial growth rates comparable to the wild type. In contrast, *GPA1* mutants in *S. cerevisiae* exhibit a severe growth defect due to activation of the pheromone response by the $G\beta\gamma$ -subunits and concomitant block in the cell cycle (Jahng *et al.* 1988). Due to the initial observation that *ste12* in *A. gossypii* are oversporulates, we analyzed sporulation of these mutants on solid medium (Table 3). While no increased sporulation was found in the G-protein subunit mutants, we identified the kinase mutants *ste11* and *ste7* as additional oversporulating strains.

In *S. cerevisiae*, components of the pheromone response pathway are part of the MAPK cascade that is induced upon starvation and signals on *Tec1* and *Ste12* (Gustin *et al.* 1998). Both transcription factors are negatively regulated by *DIG1* and *DIG2* (Chen and Thorner 2007). Deletion of *DIG1* and *DIG2* in diploids activates *Ste12* and causes a/α diploid cells to preferentially mate as a cells (Gelli 2002).

Since deletion of either *STE12* or *TEC1* resulted in a similar oversporulation phenotype in *A. gossypii*, we hypothesized that both proteins act together in transmitting upstream MAPK signals. We therefore tested whether deletion of *DIG1* (there is no *DIG2* homolog in the *A. gossypii* genome) interferes with

sporulation. Interestingly, *dig1* mutants abolish sporulation completely (Table 3). Thus our data indicate that the pheromone-response/starvation MAPK cascades in *A. gossypii* contribute to the regulation of sporulation in both positive and negative ways.

Functional analysis of sporulation specific genes

In *S. cerevisiae*, key regulatory genes in the sporulation program have been identified and homologs for most of them can be found in the *A. gossypii* genome. For our functional analysis approach, we used 15 genes (Table 3). The first set comprised six genes whose *S. cerevisiae* homologs have a function in karyogamy. We generated two independent primary heterokaryotic mutants of all strains. Via clonal selection of mutant spores, homokaryotic mutant strains can be obtained. In the case of *KAR1* and *KAR2*, however, the generation of homokaryotic strains was not possible, indicating that these genes encode essential vegetative functions similar to their *S. cerevisiae* homologs (Rose and Fink 1987; Normington *et al.* 1989; Rose *et al.* 1989). Deletion of *KAR3*, while having no growth defect at 30° showed reduced growth at 37° (Figure 2A). Thus *Kar3* is dispensable for mitotic growth. Strikingly, *A. gossypii kar3* mutants were severely reduced in sporulation. In *S. cerevisiae*, *Kar3* is required for nuclear congression to promote nuclear fusion during karyogamy (Gibeaux *et al.* 2013). This therefore suggests that a key role for *Kar3* in sporulation may be to promote karyogamy in *A. gossypii*. Furthermore, deletion of *KAR4* besides having a mild growth defect completely abolished sporulation (Figure 2, B and C). *S. cerevisiae Kar4* is a transcription factor required for karyogamy that induces *KAR3* expression (Lahav *et al.* 2007).

The second set of genes comprised homologs of key regulators of sporulation in *S. cerevisiae*, namely *IME1*, *IME2*,

Figure 3 Expression analysis of *IME2* and *IME4* during vegetative hyphal growth in *A. gossypii*. Mycelia of the *A. gossypii* strains were grown in full media and RNA was isolated during the exponential growth phase (see *Materials and Methods*). RNA was reverse transcribed and the resulting cDNA was serially diluted

(1:2 per lane from 28 ng to 0.2 ng) and used for PCR with gene-specific primers. The *A. gossypii* profilin homolog *PRF1* contains one intron and was used as quality control. Ethidium bromide-stained gels are shown, indicating lack of expression of *IME2* (left panel), while both *IME4* and *PRF1* are expressed (middle panels). The right panel shows amplification controls from DNA.

IME4, *NDT80*, and *UME6*. As expected, the *A. gossypii* mutants in these genes were either unable to sporulate or, as in the case of *ume6*, severely reduced in sporulation. Similarly to the *kar4* strain, *ime4* and *ume6* mutants showed reduced growth rates of 60–75% of the wild-type growth. The reduced growth phenotypes and the sporulation deficiencies of these strains were complemented by reintroduction of the respective wild-type genes, which in the case of *ime4* required expression from a strong promoter (data not shown, Figure 2B). A role for *IME4* during vegetative growth came unexpectedly, as *ScIME4* is not expressed in haploid yeast cells (Hongay *et al.* 2006). We analyzed the expression of *IME2* and *IME4* in growing mycelia by RT-PCR, which indicated that *IME4* is expressed under these conditions, while *IME2* is not (Figure 3).

The last gene set comprised four *A. gossypii* genes whose *S. cerevisiae* homologs have a specific meiotic function, namely *DMC1*, *SPO1*, *SPO11*, and *SPO14*. Sporulation was severely reduced in *dmc1* and *spo14* strains, reduced in *spo1*, and wild type-like in *spo11* mutants (Table 3). The sporulation levels, particularly in the *dmc1* and *kar3* mutants are reduced on solid media but are slightly improved in liquid media. In *S. cerevisiae* *Dmc1* is required for repair of meiotic DSBs (Nimonkar *et al.* 2012). Surprisingly, deletion of *A. gossypii* *SPO11*, which in *S. cerevisiae* initiates meiotic DSB formation (Keeney 2001), did not affect sporulation. To determine spore viability we micromanipulated single spores of the *dmc1*, *kar3*, *kar5*, *kar8*, *spo1*, *spo11*, and *ume6* strains and compared their ability to germinate and generate mycelia with the wild type (Table 4). Spores of the *kar5*, *kar8*, *spo1*, and *spo11* mutants basically showed wild-type vitality. Interestingly, even though sporulation of the *dmc1* strain was strongly reduced, spores that were formed were apparently as healthy as in the wild type (see *Discussion*). In contrast, fitness of the small amount of *kar3* spores that were formed proved to be rather poor. Almost 70% of *kar3* spores were either dead or produced only a germ cell that were unable to switch to polarized hyphal growth (Table 4).

Transcript profiling of sporulation-deficient mutants

We used the sporulation deficiency of the *ime1*, *ime2*, *kar4*, and *ndt80* mutants to compare their transcript profiles under sporulation-inducing conditions with that of the wild type. To this end, strains were grown in rich medium and then transferred to sporulation-inducing conditions for 16

hr. Under these conditions, the wild type will initiate the sporulation program. Incubation for 2 days or more will result in a thoroughly sporulated wild-type culture, which we wanted to avoid. To determine the transcript profiles, RNA-seq with Illumina HiSeq technology was employed. For each strain >29 mio reads were generated with 100 bases per read. The reads were aligned and annotated according to the published *A. gossypii* genome (size <10 Mb) and used for differential expression analysis (see *Materials and Methods*). Comparison of the down-regulated genes in the four mutant strains with the wild type yielded a core set of 28 genes that are down-regulated (4- to 30-fold) in all four mutant strains and 67 genes that are down-regulated in at least two mutants (Table 5). This gene set comprises a large number of *A. gossypii* homologs of *S. cerevisiae* genes known to be involved in sporulation and meiosis, which validates our analysis. Interestingly, 51 genes of this set of 67 genes was found to be up-regulated in the *ste12* mutant, providing insight into the genetic regulation of the oversporulation phenotype of this mutant (Table 5).

The expression profiles of the mutant strains also provide some insight into the underlying transcriptional network. In the *kar4* mutant, *IME1* expression is down-regulated. In the *ime1* strain, *IME2* is down-regulated, while in the *ime2* and *ndt80* strains, *IME1* is up-regulated. In the *ste12* strain, *IME1* expression is increased and *IME2* is up-regulated (Table 6). This suggests a prominent role for *IME1* as a key regulator for the sporulation program in *A. gossypii* similarly to that in *S. cerevisiae*.

ENG2 regulates hyphal fragmentation in *A. gossypii*

At the onset of sporulation, hyphal compartments separated by septa develop into sporangia. Hyphae then fragment to generate single sporangia. This step resembles the mother-daughter cell separation in yeast-like fungi (Wendland and Walther 2005). In *S. cerevisiae*, several genes code for glucanases and chitinases that are part of this separation machinery, including *CTS1*, *CTS2*, *DSE2*, *EGT2*, *ENG1*, *ENG2*, and *SCW11* (for review see Yeong 2005). Except for *CTS1* and *SCW11*, respective homologs can be found in the *A. gossypii* genome. *DSE2* is down-regulated in *kar4* and *ste12*, while *CTS2* is down-regulated only in *kar4*. Interestingly, one of the most highly up-regulated genes in *ste12* is *ENG2*, which is down-regulated in all four sporulation-deficient strains (Table 6). Since a *cts2* deletion mutant shows no defect in hyphal fragmentation (Dunkler *et al.* 2008), we

Table 4 Comparison of spore viability in *A. gossypii* mutants

	WT	<i>dmc1</i>	<i>kar3</i>	<i>kar5</i>	<i>kar8</i>	<i>spo1</i>	<i>spo11</i>	<i>ume6</i>
Mycelia	79	73	18	79	83	67	74	64
Dead	9	13	22	7	11	27	9	17
Germ cell	9	8	32	6	1	3	9	7
Abortive mycelia	3	6	6	8	5	3	8	2
N	100	100	78	100	100	100	100	90

Ungerminated spores were micromanipulated on rich medium and germination was assayed after an overnight incubation at 30°. Colony-forming mycelial outgrowth showed complete viability. Otherwise growth was not initiated (dead), stopped during the initial isotropic growth phase (germ cell stage), or stopped after a few hyphal branches had formed. Numbers represent fraction of the total number of analyzed spores (N).

analyzed the endoglucanase genes *ENG1* and *ENG2* in more detail (Figure 4). Deletion mutants in these genes do not exhibit any growth delays or sporulation defects. However, at the time when wild-type or *eng1* strains had fragmented their hyphae or set free the spores after lysis of the sporangia, we found that in the *eng2* mutant sporangia-harboring spores were still concatenated, indicating that hyphal fragmentation was inhibited or delayed. Sporangia develop from septate compartments. Septa are chitin rich and can be stained by the fluorescent dye calcofluor white. In *eng2* hyphae, the cell walls stain well with calcofluor white, yet septal sites are apparently chitin-free, suggesting that the cell wall at septal sites that connects the sporangia is mainly composed of glucan, while chitin has been degraded (Figure 4).

Up-regulated genes in the sporulation-deficient mutants

A set of 67 genes was found down-regulated in at least two strains and an additional 123 genes down-regulated in only one strain. In contrast, the overlap in the few up-regulated genes of the four sporulation-deficient strains was small. Only 1 gene turned up in three strains, ABR248W/YIR036c, a short-chain dehydrogenase/reductase, and 7 genes were up-regulated in two strains each, including the proline permease *PUT4*. Further, 58 genes were up-regulated in just one strain. Among the up-regulated genes was a set of genes encoding transport proteins, e.g., ammonium or amino acid permeases. This suggested that the sporangia in the sporulation-deficient strains are still metabolically active, e.g., in retrieval of nutrients.

To investigate if sporangia can reenter vegetative growth or are committed to the sporulation pathway and therefore locked in meiosis, we performed return-to-growth assays. To this end, mycelia were incubated in sporulation inducing minimal medium for 5 days. Although wild-type cultures are thoroughly sporulated after this incubation period, some sporangia that apparently had not formed spores could be obtained. Incubation of these sporangia for 18 hr on rich medium resulted in hyphal outgrowth in one-third of the sporangia analyzed (Figure 5). Using these conditions, eight sporulation-deficient strains were assayed for their RTG capabilities (Figure 5). Hyphal outgrowth occurred in all strains at around one-third of all sporangia. Only the *spo14* strain was less efficient with 22% RTG, while *ime1* sporangia regrew with a better than wild-type frequency. Sporangia of sporulation-

deficient strains had different phenotypes as some appeared to be highly vacuolarized. We used fluorescence microscopy to analyze the actin cytoskeleton and the vacuolar content of sporangia. We used inducing conditions as for the RTG experiment but analyzed outgrowing hyphae already after 7 hr of growth in rich liquid medium (Figure 6). Stained sporangia showed abundant cortical actin patches that were organized in a nonpolar manner across the cortex of the sporangia. Upon RTG, actin patches were localized in a polar manner to the hyphal tips. Already after 7 hr in rich medium, actin rings at sites of future septation occurred, separating the new hyphae from the sporangium (Figure 6A). Vacuoles in sporangia of all strains analyzed appeared round shape and with little motility. The sizes of the vacuoles differed from strain to strain. Yet, uptake of the lipophilic dye FM4-64 and its delivery to the vacuoles demonstrated active endocytosis under these conditions. Upon feeding of new nutrients the vacuolar compartments became more motile, changes in shape from round to ovoid occurred, and newly formed hyphal tip compartments were filled with endosomes and small vacuoles, as has previously been documented for growing *A. gossypii* hyphae (Walther and Wendland 2004). These results indicate that sporangia of either wild-type or mutant strains can reinitiate hyphal growth even after prolonged incubation in sporulation-inducing conditions.

Syntenic gene relationships at *IME1* loci

Ime1 proteins of *Saccharomycetes* vary considerably in size and amino acid sequence conservation. *ScIme1p* is 360 aa, while *E. cymbalariae* *Ime1p* is 271 aa, and *A. gossypii* *Ime1p* only 217 aa. *A. gossypii* and *E. cymbalariae* *Ime1* proteins share 59% amino acid sequence identity, whereas both of these proteins only retained <20% sequence identity to *ScIme1p*. Nevertheless, the *IME1*-encoding genes are located in syntenic loci, i.e., the gene order and transcriptional orientation of *IME1* and adjacent genes are conserved in these three species, with apparently only one addition, *AGL309W*, upstream of *AgIME1* (Figure 7). Thus, an apparent difference between *AgIME1* and the *ScIME1* and *EcymIME1* genes is the size of their promoters. The intergenic regions upstream of *ScIME1* and *EcymIME1* are >2.5 kb long, and in *S. cerevisiae* a large part of this region consists of the non-coding RNA *IRT1*. The intergenic region upstream of *AgIME1* is just 144 bp in size. However, when using this region as a promoter to drive expression of *AgIME1*, this construct did not complement the *ime1* sporulation defect, in contrast to a longer promoter that included *AGL309W* (not shown). This indicates that essential *IME1*-promoter elements must be present upstream (and within the putative ORF of *AGL309W*). *AGL309W* does not share any similarity with known genes in the databases. Upstream of *AGL309W* is *AGL310C* encoding the ribosomal protein Rpl43. The intergenic region between *RPL43* and *AGL309W* is only 26 bp, which is far too small for a bidirectional promoter. Conserved promoter elements for the transcription of ribosomal genes, i.e., binding sites for Rap1 and Fhl1, are located within the *AGL309W*-ORF. Based on

Table 5 Differential gene expression in the sporulation deficient strains *ime1*, *ime2*, *kar4*, and *ndt80* vs. the oversporulating strain *ste12*

No.	Gene	WT	<i>ime1</i>	<i>ime2</i>	<i>kar4</i>	<i>ndt80</i>	<i>ste12</i> ^a	<i>S. cerevisiae</i> gene	Fold down ^b	Fold up in <i>ste12</i>	Down + up
1	ABR127C	9326	1499	2799	842	2924	103399	YOR313C (SPS4)	4.63	11.09	15.71
2	AGL161C	7967	1429	1892	1547	1818	99078	YLR144C (ENG2)	4.77	12.44	17.20
3	AGL034C	3454	344	341	157	370	32325	No homolog	11.40	9.36	20.76
4	AGR195W	3111	366	801	836	454	25965	YBR180W (DTR1)	5.06	8.35	13.41
5	ACR122C	2857	237	575	332	374	34915	No homolog	7.53	12.22	19.75
6	ADL126C	1802	232	342	103	386	15394	YFR032C	6.78	8.54	15.32
7	AFR723C	1704	80	113	79	84	15234	YCL048W and YDR522C (SPS2)	19.15	8.94	28.09
8	ACR135C	1692	99	99	48	64	25058	YOR242C (SSP2)	21.83	14.81	36.64
9	ADL225W	1618	230	329	193	378	24839	YIL099W (SGA1)	5.73	15.35	21.08
10	AGL178W	1303	64	93	136	161	14942	YGR109W-B, YIL082W-A and YIL080W	11.48	11.47	22.95
11	AEL110W	1284	52	49	65	77	15083	YKL164C (PIR1) and YJL159W (HSP150)	21.14	11.75	32.88
12	ADR227W	1135	107	194	101	229	7077	No homolog	7.19	6.24	13.43
13	AGR175C	974	143	197	252	167	7648	YDR218C (SPR28)	5.13	7.85	12.99
14	ADL315C	933	23	28	34	32	12965	YPR054W (SMK1)	31.90	13.90	45.79
15	AFR571W	710	38	59	47	40	7111	YGR059W (SPR3)	15.43	10.02	25.45
16	AFR517C	673	81	102	112	61	9349	YLR054C	7.56	13.89	21.45
17	ACL179C	583	109	175	111	147	3807	YLR341W (SPO77)	4.30	6.53	10.83
18	AFR724C	555	41	85	81	49	6325	YDR523C (SPS1)	8.67	11.40	20.07
19	ACL182C	321	37	50	46	67	4301	YLR343W (GAS2)	6.42	13.40	19.82
20	AAL105C	308	15	17	8	19	3358	YOR255W	20.88	10.90	31.78
21	ADR078C	274	51	60	16	53	1432	YLR213C (CRR1)	6.09	5.23	11.32
22	AER235C	268	38	65	13	72	1491	No homolog	5.70	5.56	11.27
23	AFR336W	232	21	46	18	34	2365	No homolog	7.80	10.19	17.99
24	AFL213W	221	21	10	13	7	1451	No homolog	17.33	6.57	23.90
25	ADL175W	210	40	63	38	57	2332	YOL132W (GAS4)	4.24	11.10	15.35
26	ACL009C	151	33	31	27	23	901	YOL007C	5.30	5.97	11.27
27	AFR121W	121	20	48	28	32	779	No homolog	3.78	6.44	10.22
28	AGR274C	97	19	28	19	14	950	YOR177C (MPC54)	4.85	9.79	14.64
29	AER224W	12603	2435	10569	2277	1786	4156	YAR035W (YAT1)	2.95	0.33	3.28
30	AEL016C	7251	854	1266	474	4910	38271	YFR023W (PES4) and YHR015W (MIP6)	3.87	5.28	9.14
31	ABR172W	4019	658	5494	830	768	1694	YER024W (YAT2)	2.07	0.42	2.50
32	ADR123W	2995	753	1186	554	1055	17349	YBR286W (APE3)	3.38	5.79	9.17
33	ACL006W	964	286	339	270	941	3094	YMR001C (CDC5)	2.10	3.21	5.31
34	AFL028W	435	199	109	44	64	4336	No homolog	4.18	9.97	14.15
35	AER135W	335	35	92	76	123	2547	YML066C (SMA2)	4.11	7.60	11.71
36	AFR474W	282	48	109	49	538	691	YGL033W (HOP2)	1.52	2.45	3.97
37	ACL161C	249	18	102	30	367	574	YDR374C	1.93	2.31	4.23
38	ADR339C	232	36	82	41	78	1571	No homolog	3.92	6.77	10.69
39	AFL151W	231	85	100	17	37	2507	No homolog	3.87	10.85	14.72
40	AFR633W	91	21	33	37	15	798	YBR072W (HSP26)	3.43	8.77	12.20
41	ADL029W	160	34	70	57	53	712	YGL170C (SPO74)	2.99	4.45	7.44
42	AFR401W	163	30	63	71	50	1348	YDR403W (DIT1)	3.05	8.27	11.32
43	AGL351W	253	70	122	145	121	3159	YMR307W (GAS1)	2.21	12.49	14.70
44	AAL085C	258	69	124	93	462	628	YDR285W (ZIP1)	1.38	2.43	3.81
45	ABL061C-A	362	33	40	121	154	3753	YOL052C-A (DDR2)	4.16	10.37	14.53
46	AFL048W	449	131	243	229	206	1663	YBR250W (SPO23)	2.22	3.70	5.92
47	ADR121W-A	460	420	253	54	195	2994	No homolog	2.00	6.51	8.50
48	ADL165C	617	150	692	730	123	779	No homolog	1.46	1.26	2.72
49	ACR228C	622	186	309	247	507	3265	YBL063W (KIP1)	1.99	5.25	7.24
50	AEL056W	716	265	345	289	186	5351	No homolog	2.64	7.47	10.11
51	ACR287W	905	260	514	300	467	5063	YAL020C (ATS1)	2.35	5.59	7.94
52	AGR098W	1279	287	647	413	557	7688	YER180C (ISC10)	2.69	6.01	8.70
53	ADR169C	1299	307	706	411	1210	10504	YDR177W (UBC1)	1.97	8.09	10.06
54	ADR122C	1802	832	648	162	2312	7556	YAL018C	1.82	4.19	6.02
55	ADR239W	1805	519	1850	265	1852	1102	YFL041W (FET5)	1.61	0.61	2.22
56	ADL071C	2248	1076	631	127	3532	23351	YIL053W (RHR2) and YER062C (HOR2)	1.68	10.39	12.06
57	AGR235W	2676	4119	1419	319	1196	374	YIL166C, YOL163W and YOL162W	1.52	0.14	1.66
58	AGR167W	5180	1869	5708	1292	1426	10494	YGL202W (ARO8)	2.01	2.03	4.04
59	AFR076W	5440	1448	413	2505	6142	12376	YJL106W (IME2)	2.07	2.28	4.35
60	AGR003W	6616	2418	6215	275	1035	1580	YPR002W (PDH1)	2.66	0.24	2.90
61	AFR473W	6733	939	3965	658	9060	12242	No homolog	1.84	1.82	3.66

(continued)

Table 5, continued

No.	Gene	WT	<i>ime1</i>	<i>ime2</i>	<i>kar4</i>	<i>ndt80</i>	<i>ste12^a</i>	<i>S. cerevisiae</i> gene	Fold down ^b	Fold up in <i>ste12</i>	Down + up
62	AFR593C	10094	3171	15932	14926	1133	5453	YLR377C (FBP1)	1.15	0.54	1.69
63	AFR529W	14481	2958	13837	20900	472	5618	YIL162W (SUC2)	1.52	0.39	1.91
64	AFR151C	16141	8864	8972	49276	4592	10933	YLR056W (ERG3)	0.90	0.68	1.58
65	ADL062W	19877	27626	30124	5055	5213	6715	YER069W (ARG5,6)	1.17	0.34	1.51
66	ADL066C	50549	30593	64355	10686	5400	10539	YER065C (ICL1)	1.82	0.21	2.03
67	ADL065W	51986	58334	51659	9250	15185	15942	YIL057C and YER067W	1.55	0.31	1.85

^a Red-shaded values indicate down-regulated genes and orange-shaded numbers indicate up-regulated genes in comparison to wild type.

^b Fold down indicates the average fold change in the *ime1*, *ime2*, *kar4*, and *ndt80* strains. Red boxes indicate down-regulated genes; orange boxes indicate up-regulated genes in comparison to wild type.

RNAseq data, AGL309W is not expressed under sporulation-inducing conditions. Further studies are required to determine if AGL309W is expressed under vegetative growth conditions and to identify regulatory elements in the *AgIME1* promoter.

Discussion

Microbial cells base their decision to enter a developmental program that results in spore formation on environmental conditions of nutrient availability. In *S. cerevisiae* sporulation is induced by nitrogen starvation, the absence of a fermentable carbon source, but the presence of a nonfermentable carbon source (Neiman 2011). Similarly, *A. gossypii* mycelia enter the sporulation program only at the end of the growth phase, when nutrients have been depleted. The specific environmental requirements for *A. gossypii* have not been elucidated, as it readily sporulates in minimal medium, which would not be sufficient for the induction of sporulation in *S. cerevisiae*. The conserved pheromone and the starvation/filamentation MAPK cascades converge on *Ste12* and *Tec1*. A striking similarity in both *A. gossypii* and *S. cerevisiae* is the conserved role of the negative regulators *Dig1* and *Dig2*. Deletion of *DIG1* and *DIG2* enables diploid yeast cells to mate, and thus blocks sporulation. In line with this observation, deletion of *DIG1* in *A. gossypii* (which does not contain a *DIG2* gene) abolishes sporulation. In *A. gossypii* either deletion of *STE12* or deletion of *TEC1* results in an oversporulation phenotype. In this study, we have obtained the transcript profile of a *ste12* mutant and found consistent up-regulation of sporulation-specific genes. Based on their deletion phenotypes, *Ste12* and *Tec1* apparently share functions in the regulation of sporulation in *A. gossypii*, e.g., as one complex (Figure 8). In *S. cerevisiae*, the pheromone response is solely carried out by *Ste12*. Upon pheromone induction, phosphorylation of *Tec1* by *Fus3* leads to degradation of *Tec1* (Bao *et al.* 2004; Chou *et al.* 2004). However, *Ste12* and *Tec1* cooperate to induce invasive and pseudohyphal growth (Madhani and Fink 1997). *A. gossypii* represents a pre-whole genome duplication (WGD) species. Thus, the *Ste12/Tec1* cooperation may be an ancestral mechanism of gene regulation. Studies in other yeast systems will help to elucidate this transcriptional circuitry. Interestingly, in *Candida albicans*, *Tec1* was shown to be the downstream transcription factor of the pheromone response pathway of white cells, while *Cph1*, the

Ste12 ortholog, was identified as the target transcription factor of the pheromone response pathway of opaque cells (Sahni *et al.* 2010).

Karyogamy and meiosis in *A. gossypii*

Previously we have shown that mating is not a prerequisite for sporulation in *A. gossypii*, as *ste2/ste3* double mutants are sporulation competent (Wendland *et al.* 2011). This poses the question of whether karyogamy and meiosis are required to generate the haploid nuclei that are packed in spores. Here, we analyzed the function of *A. gossypii* orthologs of *S. cerevisiae* *KAR* genes, which in *S. cerevisiae* either have a function both in mitosis and karyogamy or are specifically required for nuclear fusion during mating (Kurihara *et al.* 1994). *Kar3* plays a central role in pulling yeast nuclei together to prepare for karyogamy (Gibeaux *et al.* 2013). *A. gossypii* *kar3* mutants did not reduce growth speed of fast growing mature *Ashbya* hyphae and, therefore, *Kar3* is not required for nuclear migration or positioning in vegetative hyphae. However, *kar3* mutants were strongly reduced in sporulation and the viability of spores was poor. Reduced sporulation could hint at a nuclear positioning defect within newly generated spores. However, the poor spore viability, which is in contrast to the wild-type-like spore viability of

Table 6 Comparison of the expression profiles of analyzed mutants

		WT	<i>kar4</i>	<i>ime1</i>	<i>ime2</i>	<i>ndt80</i>	<i>ste12</i>
AFR736C	<i>KAR4</i>	923	245	520	885	1192	997
AGL308W	<i>IME1</i>	636	160	109	1312	2363	1105
AFR076W	<i>IME2</i>	5440	2505	1448	413	6142	12376
AGR347W	<i>NDT80</i>	646	250	319	615	48	1288
AAL027W	<i>SPO1</i>	1544	818	592	1158	1245	3699
ADR025W	<i>SPO11</i>	80	116	74	82	122	156
AFR071W	<i>SPO14</i>	8405	10528	9364	10618	7989	6512
AGR101C	<i>DMC1</i>	1612	763	729	1419	1509	1824
AGL208C	<i>ENG1</i>	11402	5595	10659	13180	9098	5642
AGL161C	<i>ENG2</i>	7967	1547	1429	1892	1818	99078
ABL149W	<i>KAR1</i>	206	242	130	144	185	174
ACR038W	<i>KAR2</i>	38135	43720	33678	32307	47802	31451
AGR253W	<i>KAR3</i>	329	281	162	238	522	545
ADR143W	<i>KAR5</i>	91	70	87	132	101	85
ADR124C	<i>KAR8</i>	1227	828	761	1331	2146	1126

RNAseq reads per gene are indicated. Numbers in red-shaded type correspond to significantly down-regulated genes compared to the wild type, while numbers in orange-shaded type correspond to significantly up-regulated genes.

Figure 4 *A. gossypii* *ENG2* endoglucanase is required for hyphal fragmentation. The *eng1* and *eng2* mutant strains do not show growth defects when grown for 9 days on full medium plates and compared to the *leu2* parental strain (right column). Incubation in sporulation media (left and center column) results in rapid sporulation. Brightfield images show needle-shaped spores forming small clumps in *leu2* and *eng1* after 1 day and large clumps after 4 days. The *eng2* mutant under these conditions forms concatenated hyphae, which show a distinct disappearance of chitin in the cell wall at septal sites (stained with calcofluor and visualized by fluorescence microscopy). Septal sites are marked by arrows.

the poorly sporulating *dmc1* strain, suggests that an AgKar3 function in karyogamy may be conserved in *A. gossypii*.

Spo11 and *Dmc1* are yeast meiotic proteins that catalyze the formation and subsequent repair of DSBs during meiotic

recombination (Keeney 2001; Nimonkar *et al.* 2012). Interestingly, deletion of *AgSPO11* did not have an impact on sporulation in *A. gossypii*, whereas deletion of *AgDMC1* severely reduced spore formation. This suggests that *A. gossypii* can tolerate lack of DSB formation during sporulation but requires *Dmc1* to repair DSBs. In line with this is our observation that the few spores produced in a *dmc1* strain showed similar viability as the wild type, suggesting that they possibly passed through the sporulation process without the need to repair *Spo11*-induced DSBs.

Central regulators of sporulation in *A. gossypii*

In *S. cerevisiae* key regulators of the sporulation program have been identified (Mitchell 1994). Using a candidate gene approach, our analysis has identified the *IME1*, *IME2*, *IME4*, *KAR4*, *NDT80*, and also *UME6* as regulators of sporulation also in *A. gossypii*. The RNAseq data on *ime1*, *ime2*, *ndt80*, and *kar4* mutant strains identified a core set of 28 genes, whose *S. cerevisiae* homologs are all involved in sporulation. In a motif search with the intergenic promoter regions of these genes (motifsearch.com), two motifs could be retrieved; the first contains the TATA box, whereas the second has a consensus of RMCACAAAA, which corresponds to the *S. cerevisiae* *Ndt80* binding site GMCACAAAA (Ozsarac *et al.* 1997; Chu *et al.* 1998). Within the core set of down-regulated genes, seven *A. gossypii* genes were found that do not share a homolog in *S. cerevisiae*. Their role in sporulation needs to be determined. Several of these genes have homologs in other fungi: *AGL034C* encodes a protein with similarity to a secreted Cu-Zn superoxide dismutase in *C. albicans*. *AFL213W* encodes a potential acyl-CoA-dehydrogenase. *AER235C* encodes a protein with a *GCR1*-superfamily domain

Figure 5 Return-to-growth (RTG) assays in *A. gossypii* sporulation-deficient mutants. The indicated strains were grown in sporulation-inducing medium for 5 days and sporangia were plated on solid full medium to initiate return to growth. Images of the sporangia were acquired first at time point 0 right after inoculation (left panels) and again from the same sporangia after 18 hr of incubation at room temperature (right panels). The percentages of sporangia/cells able to initiate vegetative hyphal growth are listed as percentage regrowth; absolute numbers of sporangia that showed RTG vs. the number of analyzed sporangia are given in parentheses.

Figure 6 Subcellular markers for RTG in *A. gossypii*. (A) Images of the cortical actin cytoskeleton of sporangia and regrown hyphal tips of *leu2*, *ime1*, and *spo14* mutants stained by rhodamine-phalloidine. Sporangia (left panels) were grown in sporulation medium for 4 days prior to fixation and outgrown hyphae (right panels) were fixed 7 hr after inoculation into rich medium. Cortical actin patches are evenly distributed in sporangia but become tip localized in growing hyphae. Sites of future septation contain actin rings (marked by arrows). (B) Endocytosis in sporangia and regrown hyphae. Cells were incubated as in A. Live cells were stained with the lipophilic dye FM4-64 for 30 min prior to image acquisition. In sporangia vacuolar morphology is round shaped. In growing cells morphology is uneven due to vacuolar movements. Hyphal tips contain early endosomes and small vacuoles.

and may be involved in activating glycolytic enzymes, and ACR122C encodes a conserved fungal putative flavin-binding monooxygenase.

Differential regulation of *AgIME1* vs. *ScIME1*

Profound differences in the sporulation program between *A. gossypii* and *S. cerevisiae* seem to exist in the regulation of *IME1* and in upstream components (Figure 8). Haploid *S. cerevisiae* cells are blocked from entering the meiotic program by *Rme1* and the expression of noncoding RNAs. This block is removed in diploid yeast cells via the $\alpha 1/\alpha 2$ heterodimer, which represses *RME1* and *IME4*-antisense transcription (Hongay *et al.* 2006; Van Werven *et al.* 2012). The requirement of the $\alpha 1/\alpha 2$ heterodimer in *S. cerevisiae* also prohibits sporulation of illicit α/α or α/α diploids, as these cells cannot repress *RME1*.

A. gossypii filaments are a multinucleate syncytium. Cell type differences as in *S. cerevisiae* have not been observed. This may have promoted the evolutionary loss of *RME1* and altered the role of mating-type regulation on sporulation. The *A. gossypii* strain we have employed in our study har-

bors only *MATa* mating type loci (on different chromosomes). Therefore an $\alpha 1/\alpha 2$ heterodimer is not required for sporulation in *A. gossypii*. How *AgIME1* is regulated needs to be determined. Expression of *AgIME1* from the *AgTEF1* promoter is apparently detrimental and results in reduced and sectorized growth, indicating that the level of *IME1* regulation may be transcriptional (not shown).

Relaxed commitment to sporulation in *A. gossypii*

Return-to-growth studies in *S. cerevisiae* have shown that yeast cells can exit meiosis and return to mitotic growth (RTG) if nutrient limitations are relieved without the need to complete the sporulation program (Honigberg and Esposto 1994; Simchen 2009). We have used an extended incubation in sporulation medium (5 days) and still a sizeable fraction of sporangia of all mutants were able to resume hyphal growth. In fast-growing hyphae, nuclei exhibit asynchronous nuclear division cycles (Gladfelter *et al.* 2006). It is presently unknown how multiple nuclei of a single sporangium contribute to spore formation. Thus RTG in *A. gossypii* may either require exit from the sporulation program or

Figure 7 Gene order conservation at *IME1* loci. Synteny at the *IME1* locus was analyzed in *S. cerevisiae* and *E. cymbalariae* and compared to *A. gossypii*. Boxes represent genes and arrows indicate transcriptional orientation. *RPL43A* and *RPL43B* are the result of the whole genome duplication in *S. cerevisiae*. *AgRPL43* does not contain an intron indicated by the length of the arrows in the *RPL43* genes. *AGL309W* is a putative ORF that does not have a homolog in the databases. It consumes the biterminal promoter region of *AGL310C* and *AGL308W*.

With the *AGL309W*-ORF, potential DNA binding sites of the regulators of ribosomal protein transcription Abf1 (*), Fhl1 (▲), and Rap1 (●) can be found. IRT1 is a long noncoding RNA in the *ScIME1* promoter (see text).

Figure 8 Diagrammatic comparison of programs regulating sporulation in *A. gossypii* and *S. cerevisiae*. Diploid yeast cells are sporulation competent based on the repressive action of the **a1/α2** heterodimer that allows expression of *IME4* and represses *RME1*. *A. gossypii* hyphae are self-fertile. Nutrient signals in both species exert the ultimate control over sporulation as sporulation is blocked in rich medium. The core *S. cerevisiae* sporulation-specific genes are conserved in *A. gossypii* (encircled in both species).

possibly allow for an undivided nucleus to respond rapidly to changing environmental conditions and promote hyphal outgrowth.

AgENG2 promotes hyphal fragmentation

The function of one of the differentially expressed genes, *ENG2*, could be assigned to hyphal fragmentation in *A. gossypii*.

The *S. cerevisiae* *ENG2* homolog encodes an endoglucanase of the GH81 family, whose expression is strongly induced during sporulation (Baladron *et al.* 2002; Martin-Cuadrado *et al.* 2008). Interestingly, deletion of *ScENG1* results in a mother–daughter cell-separation defect, while *Sceng2* cells revealed no morphological defects either during vegetative growth or upon sporulation (Baladron *et al.* 2002). In *A. gossypii*, we identified *ENG2* as a major component of the enzymatic machinery that results in hyphal fragmentation at the end of sporulation.

Our analysis has provided a comprehensive overview of the gene repertoire engaged in different aspects of sporulation in *A. gossypii*. Transcript profiles provided further evidence that Ste12 acts as a negative regulator of sporulation in *A. gossypii*. This has opened several new research roads to elucidate, for example, the transcriptional regulation of *IME1*, a potential role of *MATα* proteins in *A. gossypii* development, or the occurrence of karyogamy and the involvement of Kar3 in this process.

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charomyces Genome Database at <http://www.yeastgenome.org/>. This research was supported in part by the European Union Marie Curie Initial Training Network Ariadne (www.eu-itn-ariadne.eu).

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