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Developmental Growth Control Exerted via the Protein A Kinase Tpk2 in *Ashbya gossypii*

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Sporulation in *Ashbya gossypii* is induced by nutrient-limited conditions and leads to the formation of haploid spores. Using RNA-seq, we have determined a gene set induced upon sporulation, which bears considerable overlap with that of *Saccharomyces cerevisiae* but also contains *A. gossypii*-specific genes. Addition of cyclic AMP (cAMP) to nutrient-limited media blocks sporulation and represses the induction of sporulation specific genes. Deletion of the protein kinase A (PKA) catalytic subunits encoded by *TPK1* and *TPK2* showed reduced growth in *tpk1* but enhanced growth in the *tpk2* strain; however, both mutants sporulated well. Sporulation can be blocked by cAMP in *tpk1* but not in *tpk2* strains. Similarly, *TPK2* acts at a second developmental switch promoting the break in spore dormancy. In *S. cerevisiae*, PKA phosphorylates and inhibits Msn2/4. The transcript profiles of the *tpk1* and *msn2/4* mutants were very similar to that of the wild type under sporulation conditions. However, deletion of the single *A. gossypii MSN2/4* homolog generated a specific sporulation defect. We identified a set of genes involved in spore wall assembly that was downregulated in the *msn2/4* mutant, particularly *DIT2*, suggesting that poor spore viability may be due to lysis of spores. Our results reveal specific functional differences between the two catalytic PKA subunits in *A. gossypii* and identified Tpk2 as the key A kinase that transduces developmental decisions of growth. Our data also suggest that Msn2/4 is involved only at a late step of sporulation in *A. gossypii* and is not a major regulator of *IME1*.

Fungi have developed remarkably complex signaling networks composed of several central conserved parts to react to changing environmental stimuli. Of key importance is the regulation of growth in response to nutrient availability, mating in response to a mating partner, or sporulation under nutrient-limited conditions. Transmembrane proteins act as sensors for external environmental stimuli and signal through conserved signal transduction pathways, including mitogen-activated protein kinase (MAPK) modules, the calcium/calcineurin pathway, and the cyclic AMP (cAMP) pathway, and have been studied in a large variety of fungi (1). Often, these pathways harbor genes required for fungal pathogenicity of plant or animal pathogens (2, 3). *Saccharomyces cerevisiae* adapts to drastically changing environmental conditions by the activation of an environmental stress response (4). This shares features of the general stress response in which Msn2/4 activate transcription of genes that harbor STRE elements in their promoters (5–7). Another immediate response to various stresses is the repression of ribosomal proteins that are regulated by Rap1 (8). Nutrient starvation in *S. cerevisiae* leads to growth arrest, reduced gene expression, and increased stress resistance (9). Interestingly, starvation of yeast cells for either glucose, nitrogen, or phosphate was found to lead to similar cAMP-dependent transcriptional changes (10).

In yeast, the cAMP/protein kinase A (PKA) pathway governs all aspects of cell physiology, particularly morphogenesis, growth, and sporulation. Adenylate cyclase is activated by either the Gpr1-Gpa2 glucose sensing pathway or by the Ras G-protein to produce cAMP. The heterotetrameric protein kinase A complex is composed of two regulatory subunits of Bcy1 and three catalytic subunits encoded in *S. cerevisiae* by *TPK1*, *TPK2*, or *TPK3*. Upon cAMP binding, Bcy1 releases the catalytic subunits. The Tpk-kinases then phosphorylate downstream effectors, which results in altered transcriptional responses (4). Activation of PKA stimulates growth and, e.g., the Rap1-dependent expression of ribosomal genes, while PKA-dependent phosphorylation of Msn2/4 leads to a downregulation of stress responses (11, 12). The influence of the level of cAMP for entry into meiosis was identified by using temperature-sensitive alleles of *CYR1* resulting in low levels of cAMP and mutants in *BCY1*, leading to constitutively active PKA. While low levels of cAMP promoted entry into meiosis, *bcy1* mutants failed to sporulate (13). Mechanistically, PKA-dependent phosphorylation of Msn2/4 leads to cytoplasmic accumulation of these transcription factors that thus cannot induce the initiator of meiosis *IME1* through binding the upstream activating sequence element IREu (see Fig. 1) (14).

Msn2 and Msn4 were considered to play largely functionally redundant roles in stress responses (15). However, nonredundant and specific roles in stress-dependent gene expression were recently found using DNA microarrays (16). Similarly, the three catalytic PKA subunits redundantly promote growth and deletion of all three kinases results in lethality (17). However, Tpk2 was found to promote pseudohyphal and invasive growth via interaction with Sfl1, whereas Tpk3 was found to inhibit these processes (18, 19). Additional diversity was found in negative regulation of

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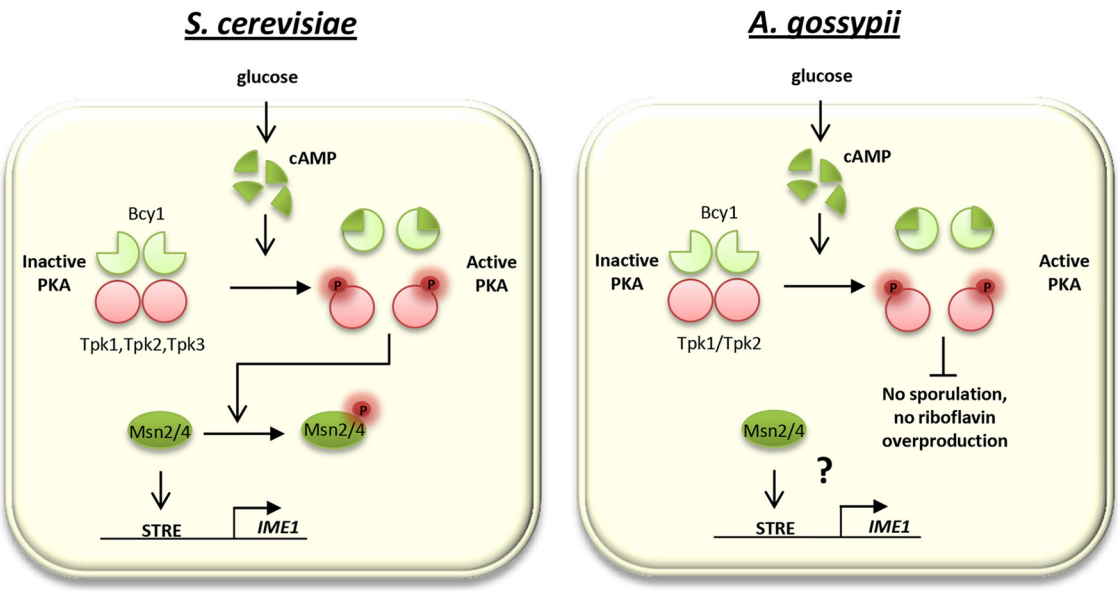

FIG 1 Glucose signaling via the cAMP/PKA pathway. A simplified overview of glucose signaling through the cAMP/PKA pathways in *S. cerevisiae* and *A. gossypii* is presented, including positive or negative regulatory functions and their potential link to the Initiator of Meiosis, *IME1*.

iron uptake by Tpk2, which also promotes trehalose degradation. Interestingly, *tpk2* mutants grow better on ethanol-glycerol-containing media than do wild-type and *tpk1* or *tpk3* strains (20). The gene set upregulated during yeast sporulation has been determined using DNA microarrays. Temporal patterns were observed to classify early, middle, and late induction of gene expression under nutrient-limited conditions (21).

Ashbya gossypii is a distant relative of *S. cerevisiae* that solely grows in a hyphal form. Upon nutrient limitation, *A. gossypii* mycelia initiate developmental events leading to the formation of sporangia, hyphal fragmentation, sporulation, and riboflavin overproduction (22, 23). Previously, conserved roles of key *S. cerevisiae* genes regulating meiosis, e.g., *IME1*, *IME2*, *IME4*, and *NDT80*, for the sporulation in *A. gossypii* were identified (24). Similar to *bcy1* mutants in yeast, exogenous addition of cAMP to the growth medium promoting constitutive activation of PKA blocks sporulation in *A. gossypii* (25).

To elucidate the function of cAMP signaling in sporulation, we compared the transcriptional profiles of the wild type under nutrient-rich, nutrient-limited, and nutrient-limited/cAMP-supplemented conditions to identify the gene set upregulated under sporulation-inducing conditions. We found that a gene set comprising 560 genes is 5-fold upregulated under sporulation inducing conditions, most of which were downregulated when cAMP was added to nutrient-limited medium. To identify functional differences between the catalytic PKA subunits, we analyzed *TPK1* and *TPK2* mutants. Strains with *TPK2* deleted grew faster than the wild type but were defective in cAMP-dependent repression of sporulation. In contrast, *tpk1* strains responded like the wild type to cAMP with repressed sporulation in nutrient-limited media. On the other hand, spore germination was severely reduced in *tpk2* strains. As a potential PKA target gene in sporulation, we analyzed *A. gossypii msn2/4*. The transcript profile of *msn2/4* was very similar to those of wild-type and *tpk1* strains. However, a small set of genes involved in spore wall formation, e.g., *DIT2*, was downregulated in *msn2/4* under nutrient-limited condition. A role for Msn2/4 in spore wall maturation is supported by a low level of viable spores in *msn2/4* strains.

MATERIALS AND METHODS

Strains and media. *A. gossypii* strains were grown in *Ashbya* full medium (AFM; 1% yeast extract, 1% peptone, 2% dextrose), and G418/Geneticin (200 μg/ml) was used for selection of antibiotic-resistant transformants. For sporulation, overnight cultures of *A. gossypii* adjusted to similar wet weights were further incubated in minimal medium (1.7 g/liter yeast nitrogen base [YNB] without ammonium sulfate and without amino acids, 0.79 g/liter Bio 101 complete synthetic mixture, 20 g/liter glucose, 2 g/liter asparagine, and 1 g/liter *myo*-inositol) for up to 3 days. Residual mycelia were digested with zymolyase, and spores were suspended in spore buffer (0.03% Trition-X-100). *A. gossypii* strains were grown at 30°C unless stated otherwise. *Escherichia coli* strain DH5α was used for plasmid propagation and grown at 37°C with ampicillin for the selection of antibiotic-resistant transformants.

Generation of plasmids and gene fusions. Disruption cassettes for *TPK1* and *TPK2* were obtained by cloning 5′ and 3′ homologous flanks to the *GEN3* selectable marker. The 5′ flanking regions were amplified using the primer pair 1818/1819 for *TPK1* and the primer pair 1822/1823 for *TPK2*. SacI sites were included in primers 1818 and 1822, and BamHI sites were included in primers 1819 and 1823. The 3′ flanking regions were amplified by using the primer pair 1820/1821 for *TPK1* and the primer pair 1824/1825 for *TPK2*. BamHI sites were included in primers 1820 and 1824, and EcoRI sites were included in primers 1821 and 1825. The *GEN3* marker was excised from pFA-GEN3 using BglII and cloned into the BamHI site between the 5′ and 3′ flanking regions of each gene into pBluescript SK(+). The *GEN3* marker was inserted in the reverse orientation in the *TPK1* disruption cassette (pSK-tpk1::GEN3) and in the forward orientation in the *TPK2* disruption cassette (pSK-tpk2::GEN3). Oligonucleotides were obtained from Integrated DNA Technologies (Leuven, Belgium).

Transformation of *A. gossypii*. *A. gossypii* strains were transformed by electroporation using *GEN3* as selectable marker, providing resistance to the antibiotic G418 as described previously (26). For each desired deletion mutant, two independent transformants were generated. The *A. gossypii msn2/4* deletion strains were generated by PCR-based gene targeting as described previously (26). The *A. gossypii tpk1* and *tpk2* deletion strains

were generated by transformation with cloned disruption cassettes obtained by digesting pSK-tpk1::GEN3 and pSK-tpk2::GEN3 with SacI. Diagnostic PCR was used to verify the correct integration of a disruption cassette and the deletion of the target gene in two independent homokaryotic deletion strains as described previously (27).

Microscopy. Microscopy was carried out using a Zeiss Axio Imager M1 microscope (Zeiss, Jena, Germany) controlled by Metamorph 7 software (Molecular Devices Corp., Downington, PA). Images were acquired with a Photometrics Coolsnap HQ camera (Princeton Instruments, Trenton, NJ). To monitor the germination of mutant spores, an Axiovert 200M microscope was used.

Determining sporulation ability. Sporulation efficiency was assayed in both homokaryons and heterokaryons for each mutant strain by isolating spores from the central part of mycelia grown for 10 days at 30°C on AFM plates supplemented with 1 g/liter myo-inositol. A circle of the central mycelia (15 mm in diameter) was cut out from the plate and suspended in 5 ml of Tris-EDTA buffer containing 200 μl of zymolase to degrade cell walls of vegetative mycelia and release the spores. After 3 h of incubation at 37°C (on a tilting rotor), the spores were collected by centrifugation and washed twice in spore buffer (0.03% Triton X-100). For determination of the CFU, serial dilutions were performed in spore buffer, and 100 μl of appropriate dilutions was plated on AFM plates, followed by incubation at 30°C until colonies appeared. The data are presented as the percent CFU forming in the homokaryon compared to the heterokaryon of each deletion strain.

Germination efficiency. Spores were isolated from mycelia grown in sporulation media for 3 days. A total of 100 spores of the wild-type strain and the *tpk2* deletion strain were micromanipulated using a Singer MSM 300 dissection microscope (Singer Instruments, Somerset, United Kingdom) and grown on AFM plates at 30°C up to for 3 days. Events scored (including by microscopy) were ungerminated spores, spores that only formed germ cells, spores that germinated but generated abortive mycelia, and spores that developed mycelia.

RNA-seq. Mycelia of two independent homokaryons for each deletion strain were grown in liquid AFM medium in baffled flasks for 16 h. Equal wet weights of mycelia were washed and grown either in AFM or under nutrient-limited conditions in minimal media for 16 h prior to total RNA isolation with a RiboPure-Yeast kit (Life Technologies). The RNA quality was assayed photometrically and by performing reverse transcription-PCR on selected target genes. To study the influence of cAMP on gene expression under nutrient-limited conditions, 5 mM cAMP was added to the minimal medium. The total RNA was used for Illumina HiSeq 2000 sequencing, and data were processed to remove linker DNA, rRNAs, and tRNA sequences. Short reads were discarded so that 16.5 to 28.0 million reads were obtained per strain. Reads were aligned, and read counts were generated for each annotated gene (using TopHat 2.0.4) based on the published *A. gossypii* genome. Differential expression based on the RNA-seq data was analyzed as described previously using edgeR, DESeq, and cufflinks, and a false discovery rate of <0.05 was indicative of differential expression (24). RNA-seq was performed by LGC Genomics (Berlin, Germany).

GO-term analyses were carried out using the Generic Gene Ontology (GO) Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder) or the GO Term Finder at the *Saccharomyces* Genome Database (http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl).

RESULTS

The *Ashbya gossypii* transcriptome under nutrient-limited conditions. *A. gossypii* produces abundant amounts of spores due to mycelial fragmentation and near quantitative conversion of hyphae into sporangia, which is much in contrast to its close relative, *Eremothecium cymbalariae* (28). *A. gossypii* spores are haploid and uninucleate, suggesting that the resulting mycelium formed by a single spore is homothallic since it is itself able to sporulate (29, 30). Previous RNA-seq analyses focused on a comparison of the transcriptomes of nonsporulating *A. gossypii* mutants, e.g., *ime1* and *ime2* strains, with the wild type (24).

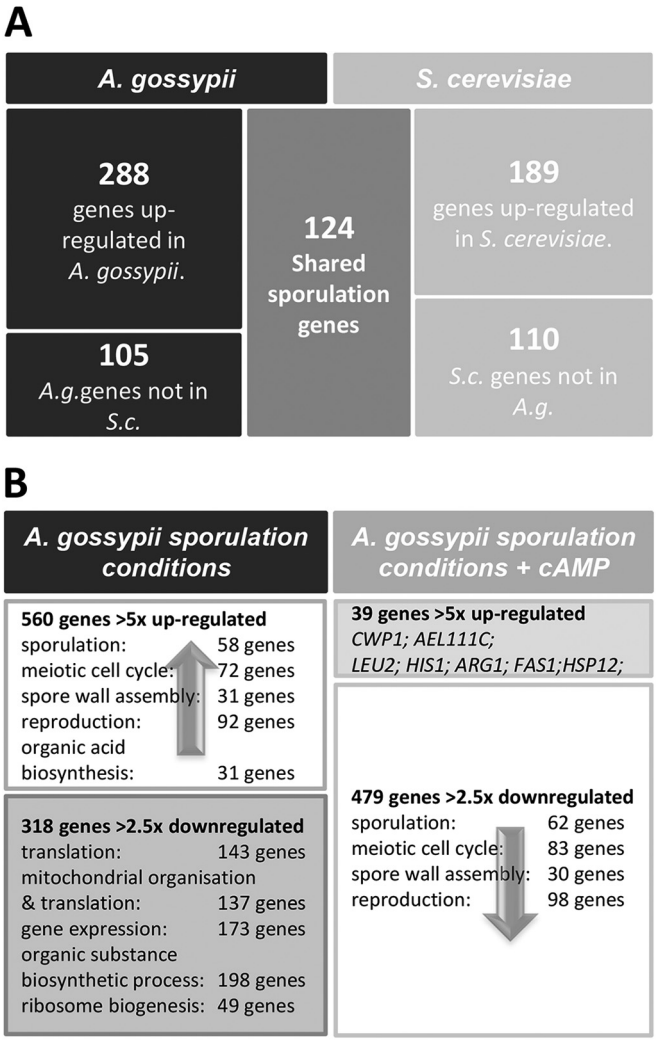

FIG 2 RNA-seq transcription profiling under nutrient-limited conditions in *A. gossypii*. (A) Comparison of upregulated genes under nutrient-limited conditions in *A. gossypii* and *S. cerevisiae* (based on reference 5). The number of genes indicated in the center are upregulated in both species, the number of genes to the left and right are regulated in a species-specific manner. (B) Based on GO term analyses specific GO IDs were identified as either up- or down-regulated. The left part indicates processes regulated during sporulation in minimal medium, and the right part indicates processes regulated in minimal medium that was supplemented with 5 mM cAMP.

In the present study, we sought to identify the set of *A. gossypii* genes that is differentially expressed under nutrient-limited conditions that results in sporulation. Wild-type mycelia were transferred from complete medium to either minimal or complete medium and incubated for 16 h. This is sufficient to induce sporulation in *A. gossypii*. Total RNA was isolated and used for RNA-seq transcript profiling. All experiments were conducted by pooling RNA of two independent transformants per assayed mutant. The complete data set is included as File S1 in the supplemental material. We considered genes upregulated under these conditions as part of the transcriptional program of sporulation in *A. gossypii*. Using a cutoff 5-fold overexpression, we identified 559

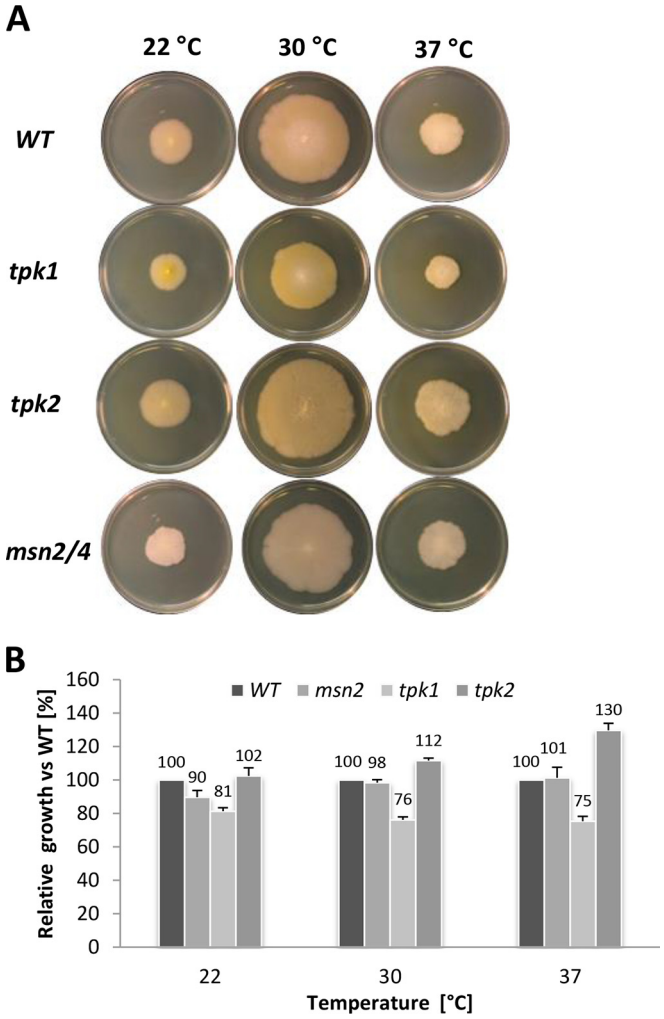

FIG 3 Role of components of the cAMP/PKA pathway for vegetative growth. (A) Comparison of radial growth rates of the wild-type strain and the *tpk1*, *tpk2*, and *msn2/4* mutants at different temperatures. (B) Bar diagram comparing the radial growth of the mutants with the wild type after 7 days at 22, 30, and 37°C. Strains were grown on complete media plates, and the radial growth was measured after 7 days and is presented as the percent growth of the wild type. The bars represent duplicate experiments of the independent homokaryotic mutant strains for each gene.

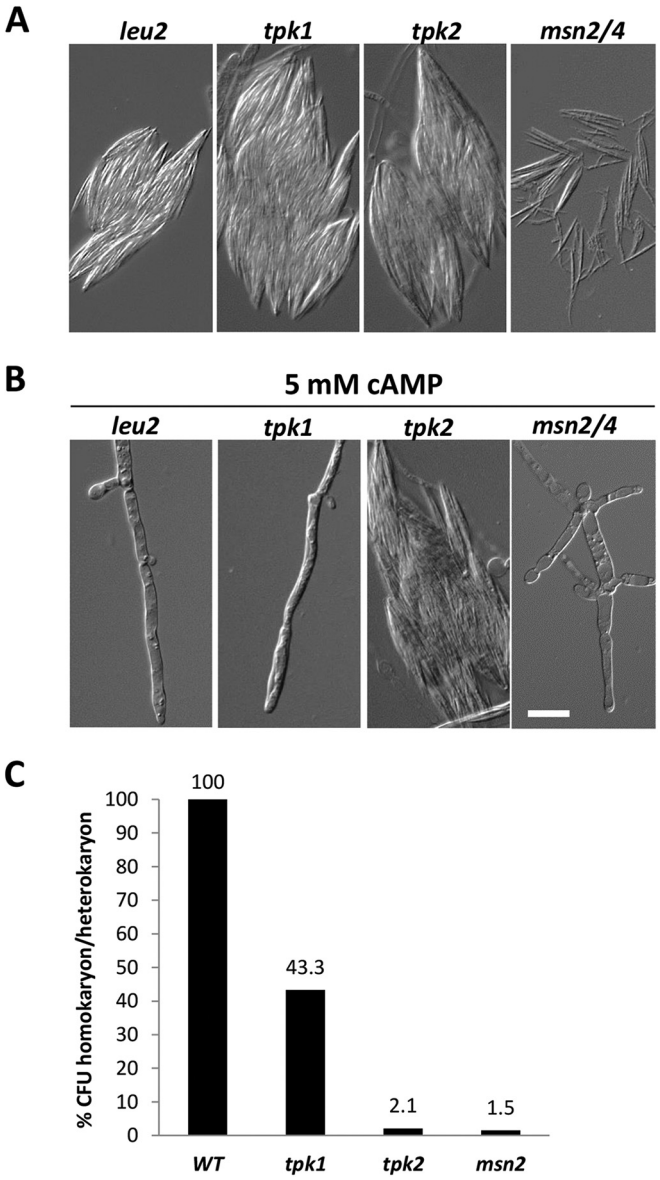

FIG 4 Sporulation ability of the *tpk1*, *tpk2*, and *msn2/4* mutants. (A and B) Sporulation ability of the indicated strains with or without the addition of 5 mM cAMP is shown by the presence of spore clumps or hyphae. Note the difference in spore shape and appearance of *msn2/4* spores compared to spores of the other strains. (C) Chart showing the efficiency of colony formation from spores derived from the indicated strains. Wild type was set to 100%. For the mutant strains, the ratio was calculated based on a comparison of heterokaryotic and homokaryotic strains. The strains in panels A and B were grown for 3 days in liquid sporulation media. Then, spore suspensions were treated with zymolyase to digest the remaining cell walls from vegetative hyphae. Representative images derived from duplicate experiments of independent homokaryotic mutant strains for each gene are shown. Scale bar, 10 μm.

genes that included 454 genes with homologs in *S. cerevisiae*. This included some genes that are duplicated in *A. gossypii*. This gene set was compared to the set of genes upregulated during sporulation in yeast (21). A core set of 124 sporulation induced genes was found in both species (Fig. 2A and see File S1 in the supplemental material). Among these upregulated genes, for example, were the transcriptional regulators *IME1* (upregulated 460-fold), *IME2* (54-fold), and *NDT80* (108-fold), the middle-sporulation specific MAPK *SMK1* (1,500-fold), and the sporulation-specific septins *SPR3* (1,950-fold) and *SPR28* (120-fold). Genes for spore wall formation, such as *DTR1* (806-fold), *GAS2* (777-fold), *GAS4* (666-fold), *OSW1* (647-fold), *DIT1* (496-fold), and *DIT2* (306-fold), were also found to be highly upregulated. This provides an indication on the progress of the developmental process in *A. gossypii* mycelia within a 16-h period between a shift from complete to minimal medium. With just one sampling time point, we, of course, could not distinguish between an early, a middle, or a late phase of induction of these genes. Interestingly, a large set of homologous genes seems to be differentially upregulated in sporulation in *A. gossypii* or in *S. cerevisiae*. At the same time, ~100 genes were found to be upregulated during sporulation in each of the two species for which there was no homolog in the other species (Fig. 2A). Some of these genes (termed "NOHBY" since they

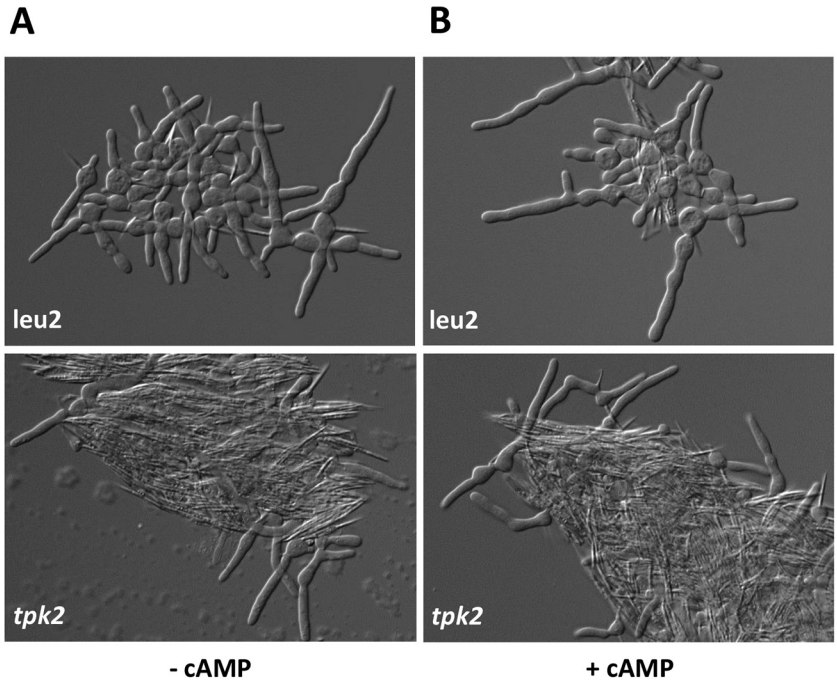

FIG 5 Germination defect of *tpk2* spores in liquid medium. Microscopic images of spores of the wild-type and *tpk2* strains that were germinated in complete liquid medium overnight at room temperature without (A) or with (B) the addition of 5 mM cAMP to the growth medium are shown.

have "NO Homolog in Baker's Yeast" [30]) were actually upregulated to a high level and will make interesting candidates for future studies.

We used the set of 454 *A. gossypii* genes upregulated under nutrient-limited conditions that share a *S. cerevisiae* homolog and queried the GO database (http://go.princeton.edu/cgi-bin/GOTermFinder) in order to find genes belonging to the same process (Fig. 2B). As expected, the upregulated genes belong to processes of meiosis, sporulation, and spore wall formation. Among the downregulated genes, translation and gene expression were the most abundant (see Table S1 in the supplemental material).

cAMP blocks sporulation specific gene expression in *A. gossypii*. In *A. gossypii*, the exogenous addition of cAMP blocks sporulation and riboflavin overproduction (25). We thus added cAMP to a nutrient-limited culture to identify the cAMP-repressed gene set (Fig. 2B). We identified 479 genes that were >2.5-fold downregulated, and the GO terms were exactly those that were upregulated under nutrient-limited conditions, i.e., genes involved in sporulation. Specifically, the expression levels of *IME1*, *IME2*, *IME4*, and *NDT80* were strongly reduced by the addition of cAMP. This indicates that the cAMP/PKA pathway controls all developmental events leading to sporangium formation and sporulation. However, we did not find a cAMP repression of any of the riboflavin biosynthesis genes under nutrient-limited conditions. Only a small set of genes was found to be upregulated under these conditions, including *CWP1* and AEL111C (YKL164C) involved in cell wall stability or genes involved in amino acid biosynthesis (Fig. 2B).

Specific signaling functions of *TPK1* and *TPK2* during growth and sporulation. Due to the central role of PKA for development in *A. gossypii*, we wanted to determine whether the catalytic subunits encoded in *A. gossypii* by *TPK1* and *TPK2* are redundantly regulating sporulation or harbor specific functions. The *tpk1* and *tpk2* mutant strains were assayed for radial growth speed on complete medium at different temperatures (Fig. 3A). Strains with *TPK1* deleted showed ca. 25% reduced growth at 30 and 37°C, while *tpk2* strains grew faster than the wild type at these temperatures (Fig. 3B). This suggests specific roles for Tpk1 and Tpk2 in hyphal growth (see Discussion).

Both *tpk1* and *tpk2* strains were able to sporulate and, in fact, produced a large amount of spores (Fig. 4A). Since the addition of cAMP blocks sporulation in the *A. gossypii* wild type under nutrient-limited conditions, we tested whether this development is also repressed by cAMP in *tpk1* and *tpk2* strains. We found that, as in the wild type, sporulation was repressed by the addition of cAMP in *tpk1* mutant strains. Surprisingly, however, *tpk2* strains were unresponsive to cAMP addition and sporulated abundantly (Fig. 4B). This indicates that Tpk2 acts as a gate keeper to control development and initiate sporulation by transducing nutrient signals to downstream effectors.

Spores of *tpk2* strains exhibit a severe germination defect. To determine the viability of the *tpk1* and *tpk2* spores, we purified spores by digesting mycelial fragments with zymolyase and plating them on complete media to determine the number of CFU (Fig. 4C). With the wild type set to 100%, we compared the CFU of *tpk1* and *tpk2* homokaryotic versus heterokaryotic strains. The heterokaryotic *TPK/tpk* strains served as a control for assessing the recessive sporulation defect. We found that the deletion of *TPK1* reduced the ability of spores to form colonies by half since 43.3% of the homokaryotic spores formed CFU compared to the heterokaryotic spores. However, *tpk2* spores were much more severely reduced in their ability to generate mycelia—only 2.1% CFU in the homokaryon versus the heterokaryon—even though spores

TABLE 1 Reduced germination efficiency of *tpk2* spores

Spore type	Germination efficiency (no. of spores)	
	Wild type	*tpk2* mutant
Mycelia	58	11
Ungerminated	22	89
Germ cell	8	0
Abortive mycelia	12	0
Total	100	100

appeared to be wild type-like. We here discovered a key role of Tpk2 to govern the developmental switch between growth and sporulation. The poor ability to generate mycelia from spores led us to investigate a second developmental switch: that of breaking the dormancy in a spore to promote germination and hyphal growth.

To assess whether the inability to form colonies was due to a germination defect or, e.g., postgermination lysis of mycelia, we inoculated spores of the wild-type and *tpk2* mutant strains into liquid complete medium. Wild-type spores readily germinated and formed juvenile mycelia by 16 h after inoculation. In contrast, *tpk2* spores remained largely ungerminated, and only a few mycelia developed (Fig. 5A). The germination efficiency of the *tpk2* spores was quantified by micromanipulation of 100 spores each from the wild-type and *tpk2* strains on full medium plates. Only ca. 10% of *tpk2* spores germinated and formed mycelia, whereas almost 80% of wild-type spores germinated, even though 20% of the spores did not mature into mycelia (Table 1). We also observed a large germination delay in *tpk2* spores in comparison to the wild type (not shown).

To investigate whether the block in germination could be suppressed by additional supplementation with cAMP to promote breaking the dormancy of *tpk2* spores, e.g., by overactivating Tpk1, wild-type and *tpk2* spores were germinated in liquid and solid complete media supplemented with cAMP (Fig. 5B and 6). However, cAMP-addition did not improve the germination rate of *tpk2* spores and also had no effect on wild-type or *tpk1* spores (data not shown).

Deletion of *MSN2* results in low spore viability and downregulation of spore wall biosynthetic genes. Two of the key downstream effectors of PKA in *S. cerevisiae* are Msn2 and Msn4 (31, 32). *A. gossypii* has one homolog denoted *MSN2/4*. Deletion of *MSN2/4* does not result in a growth defect (Fig. 3A). Deletion mutants of *MSN2/4* are able to sporulate, and this development can be suppressed by the addition of cAMP to nutrient-limited medium (Fig. 4A and B). However, we observed a low rate of colony formation from *msn2/4* spore preparations (Fig. 4C). Microscopic inspection of the spores showed that spores were often broken and were of granulated appearance ("ghost-like"; Fig. 4A). The RNA-seq transcript profile of *msn2/4* strains under sporulation conditions was mostly congruent with those of the wild-type and *tpk1* mutant strains (see File S1 in the supplemental material). However, we observed a specific downregulation of genes involved in spore wall formation when comparing the *msn2/4* strain to the wild type under sporulation conditions, particularly that of *DIT2* (Table 2). Even though the genes listed in Table 2 demonstrated an upregulation compared to wild type grown in full medium, they nevertheless were induced to a far lesser level (only one-third) compared to the wild type in nutrient-limited medium. This suggests that poor cell wall formation reduces spore viability, which may be exacerbated when these spores are treated with zymolyase during preparation.

DISCUSSION

Key decisions on cell fate in fungi are gated to control metabolism, stress response, pathogenicity, growth, and developmental switches for entering quiescence, sporulation, or breaking the dormancy of spores (2, 33, 34). Signal transduction cascades play an essential role to parse environmental information and result in appropriate cellular responses (1). Nutrient availability is signaled through the cAMP/PKA pathway to promote growth and developmental decisions (35). Changes in metabolism often require large-scale transcriptional changes affecting hundreds of genes. In *S. cerevisiae* such large-scale changes were identified under various stress conditions, nutrient limitation, and sporulation (4, 10, 21, 36, 37).

We have shown that in *A. gossypii* sporulation leads to differential expression of ~900 genes, including the upregulation of conserved genes involved in sporulation, meiosis, and spore wall assembly and the downregulation of genes involved in translation and gene expression. Although there is considerable overlap with the transcriptional program of *S. cerevisiae* sporulation, there are several hundred genes specifically upregulated in *A. gossypii*, including 100 genes that do not share a homolog with yeast. These genes, particularly the most highly upregulated ones, could be informative to elucidate differences of spore shapes in *A. gossypii* (needled-shaped) versus *S. cerevisiae* (round spores).

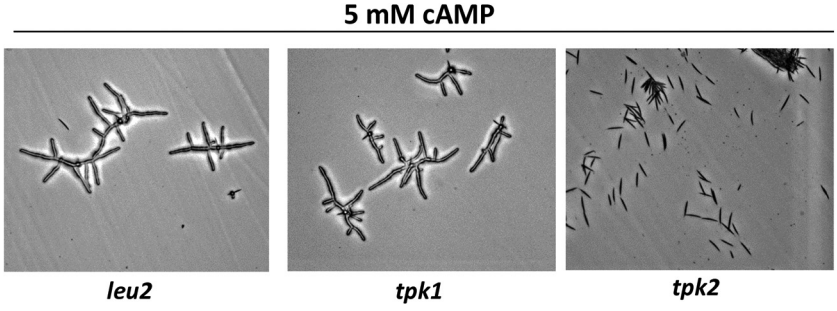

FIG 6 Germination defect of *tpk2* spores on solid medium. The germination defect of *tpk2* spores cannot be suppressed by growth on solid medium. Microscopic images of spores of the wild-type and the *tpk1* and *tpk2* strains that were plated on complete medium and grown overnight are shown.

TABLE 2 Genes involved in spore wall formation, which are downregulated in the *msn2/4* strain versus the wild type under sporulation conditions

A. gossypii gene	*S. cerevisiae* homolog	Gene	No. of reads[a]			Fold downregulation (*msn2/4* vs *leu2*/spo)
			leu2/spo	*leu2*/AFM	*msn2/4*/spo	
AFR400C	YDR402C	*DIT2*	2,455	8	372	–6,60
AFR401W	YDR403W	*DIT1*	31,248	63	7,103	–4,40
ACL102W	YIR026C	*YVH1*	190	293	51	–3,73
AGR195W	YBR180W	*DTR1*	105,271	129	32,183	–3,27
ACR135C	YOR242C	*SSP2*	88,026	69	27,567	–3,19
ADL315C	YPR054W	*SMK1*	48,690	32	15,460	–3,15
AFR517C	YLR054C	*OSW2*	53,481	38	17,029	–3,14
ACL182C	YLR343W	*GAS2*	30,309	39	9,709	–3,12
AFR723C	YCL048W	*SPS22*	56,780	42	18,572	–3,06
AAL105C	YOR255W	*OSW1*	11,005	17	3,793	–2,90
AGR105C	YDR104C	*SPO71*	65,188	627	22,777	–2,86
AFR724C	YDR523C	*SPS1*	30,126	33	10,547	–2,86
ABL089W	YBR045C	*GIP1*	32,406	38	11,463	–2,83
AGR274C	YOR177C	*MPC54*	4,862	4	1,722	–2,82
ADL105C	YER046W	*SPO73*	2,640	62	944	–2,80
ACL179C	YLR341W	*SPO77*	24,172	83	8,714	–2,77
ADL135C	YGR225W	*AMA1*	20,387	589	7,562	–2,70
AFR524W	YOR298W	*MUM3*	2,632	51	983	–2,68
AFR604C	YOL091W	*SPO21*	30,580	611	11,602	–2,64
AAR069W	YCR045C	*RRT12*	20,571	544	8,103	–2,54

[a] The total numbers of reads obtained by RNA-seq expression profiling of the parental strain under sporulation conditions (*leu2*/spo strain), the parental strain in complete AFM medium (*leu2*/AFM), and the *msn2/4* strain under sporulation conditions (*msn2*/spo) are shown.

The addition of cAMP to nutrient-limited conditions resulted in the downregulation of genes involved in translation comparable to the wild type without the addition of cAMP, and yet it specifically inhibited the expression of sporulation-specific genes and effectively blocked the sporulation in *A. gossypii*. Overactivation of cAMP signaling, e.g., by deletion of the regulatory subunit of PKA or by overexpression of the catalytic subunit, was shown to result in reduced conidiation in *Aspergillus fumigatus* and *Alternaria alternate* (38–40). Similarly, deletion of genes encoding phosphodiesterases, which hydrolyze cAMP, resulted in drastically reduced conidiation in *Botrytis cinerea* and *Magnaporthe oryzae* (41, 42).

Of great interest has been the regulation of pseudohyphal growth in *S. cerevisiae* upon starvation for nitrogen (18, 43). Particularly, diverged and specific functions in yeast pseudohyphal growth were discovered with Tpk2 promoting it, whereas Tpk3 inhibits it (19). Morphogenetic decisions often are accompanied with virulence. Accordingly, the PKA pathway was also identified as a key regulator of fungal pathogenicity (2). In *Candida albicans* Tpk2 regulates dimorphism, and *tpk2* strains are crippled in their ability to invade tissues and thus reduced in virulence (44). Contact-induced appressorium formation and host penetration in *M. oryzae* and morphogenesis and pathogenicity in *Ustilago maydis* require signaling via the cAMP/PKA pathway (45).

We have shown that the catalytic PKA subunits of *A. gossypii* have decisively distinct functions in transducing the cAMP signal to affect developmental decisions. *TPK1* deletion affects vegetative growth but not developmental decisions in *A. gossypii*. In contrast, Tpk2 is required at key developmental transitions. On the one hand, Tpk2 is essential for blocking sporulation when cAMP levels are high, which signals abundant nutrient supply. *TPK2* deletion mutants are completely defective in blocking this developmental step and simply override this block. On the other hand, Tpk2 is needed for the initiation of germination to break the dormancy of spores. *TPK2* mutants show a severe reduction in germination efficiency. At both of these stages, signals of favorable growth conditions and abundant nutrient supply are not relayed in *tpk2* to promote growth, identifying Tpk2 as a key gatekeeper of developmental decisions in *A. gossypii*. Interestingly, when Tpk2 is the sole A kinase, i.e., in the *tpk1* mutant strain, radial growth rate is reduced. This suggests an inhibitory function of Tpk2, resulting in submaximal radial growth rate. Conversely, deletion of *TPK2* leads to increased growth rates. This, therefore, indicates a third specific function for Tpk2, distinguishing it further from Tpk1.

Sensitivity of germination to cAMP signaling has also been reported in *Aspergillus nidulans*. Here, carbon source signaling is relayed via the heterotrimeric G protein GanB(α)-SfaD(β)-GpgA(γ) to PKA. The deletion of GanB results in a severe germination defect (46). Similarly, deletion of the catalytic PKA subunits *pkaC1* and *pkaC2* in *A. fumigatus* resulted in delayed germination (47).

One of the functions of PKA is to regulate stress responses via Msn2/4 (32). We have previously studied regulation of sporulation in *A. gossypii* and described a sporulation defect of *ime1* and *ime2* mutants (24). To address whether *MSN2/4* could establish a link between PKA signaling and *IME1* expression, we generated *MSN2/4* deletion strains. These strains, however, showed transcript profiles similar to that of the wild type under nutrient-limited conditions and produced spores. Nevertheless, spore viability was drastically reduced in *Agmsn2*/4 strains. We have provided evidence that this may be due to the downregulation of genes involved in spore wall formation, e.g., *DIT2*. A spore wall defect of *msn2/4* mutants was recently also described for *S. cerevisiae* (48). Thus, our current research aims at identifying other more potent links connecting the cAMP/PKA pathway with, e.g., *IME1* and induction of the transcriptional program of sporulation in *A. gossypii*.

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