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Analysis of the cell wall integrity pathway of *Ashbya gossypii*

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ABSTRACT

Fungal cells are exposed to rapidly changing environmental conditions, in particular with regard to the osmotic potential. This requires constant remodeling of the cell wall and, therefore, the cell wall integrity (CWI) MAP-kinase pathway plays a major role in shaping the fungal cell wall to protect from adverse external stresses. To provide a comprehensive functional analysis of the *Ashbya gossypii* CWI pathway we generated a set of ten deletion mutants in conserved components including the cell surface sensors *AgWSC1* and *AgMID2*, a putative Rho1-guanine nucleotide exchange factor, *AgTUS1*, the protein kinase C, *AgPKC1*, the MAP-kinases *AgBCK1*, *AgMKK1* and *AgMPK1*, and transcription factors known to be involved in CWI signaling *AgRLM1*, *AgSWI4* and *AgSWI6*. Deletion of *AgPKC1* shows a severe growth defect with frequent tip cell lysis. Deletion of components of the MAP-kinase module generates a pronounced colony lysis phenotype in older regions of the mycelium. Cytoplasmic leakage was assayed using alkaline phosphatase and β-galactosidase release assays. This indicated that the lysis phenotypes of CWI pathway mutants may be useful to facilitate the isolation of riboflavin from *A. gossypii*. Remarkably, the *Agwsc1* mutant showed a strong (up to 8-fold) increase of riboflavin in the growth medium compared to the parental strain

Introduction

The cell wall integrity (CWI) signaling pathway is required for remodeling of the cell wall during growth and upon cell wall stress in fungi (Fuchs and Mylonakis 2009). The diverse natural environments that fungi inhabit may generate a variety of stresses that must be dealt with, including changes in osmolarity, temperature, and pH. These stresses will primarily act on the fungal cell wall, which generates a need for remodeling, reinforcement, or repair. For example, treatment of *Candida albicans* with caspofungin, which inhibits the cell wall β-1,3-glucan synthase, leads to an increased chitin content of the cell wall (Walker et al. 2013). On the other hand, cell growth requires adding cell wall material at sites of growth either in a cell cycle dependent manner for yeast cells or focussed at the hyphal tip for filamentous fungi.

The CWI pathway has been studied in great detail in *Saccharomyces cerevisiae* (Levin 2005, 2011). Extracellular stresses are transduced into the cell by a set of cell wall sensors, including Wsc1 and Mid2 (Verna et al. 1997; Ketela et al. 1999). This activates Rho1 *via* its guanine nucleotide exchange factor Rom2 and leads to activation of Pkc1, which in turn triggers a MAP kinase module consisting of Bck1, Mkk1, and Mpk1/Slt2 (Banuett 1998). This cascade terminates at several transcription factors including Swi4, Swi6, and Rlm1 (Watanabe et al. 1995; Fuchs and Mylonakis 2009; Levin 2011). The CWI pathway is highly conserved in fungal species and the corresponding homologs are therefore also present in *Ashbya gossypii* (Rispail et al. 2009). Previously, the *RHO1* paralogs *RHO1a* (*RHOH*) and *RHO1b* (*RHO1*) have been shown to be involved in the maintenance of cell wall integrity in *A. gossypii*, with *RHO1b* playing the major role (Wendland and Philippsen 2001; Walther and Wendland 2005; Kohli et al. 2008).

At the end of the growth phase *A. gossypii* hyphae prepare for sporulation. Onset of sporulation results in sporangium development and hyphal fragmentation. Lysis of sporangia releases the spores (Wendland and Walther 2005). *A. gossypii* is a flavinogenic fungus that is used industrially for the production of riboflavin (Stahmann et al. 2000). Riboflavin production increases concomitant with sporulation and it has been suggested that riboflavin protects *A. gossypii* spores from UV-damage (Stahmann et al. 2001; Abbas and Sibirny 2011). Riboflavin is not only secreted into the medium but is also stored in the vacuole. Elimination of vacuolar retention by deletion of the *VMA1* gene led to the quantitative secretion of riboflavin into the growth medium (Forster et al. 1999). The latter study indicated that apart from overproduction a key step to maximize riboflavin yield is the ability to harvest riboflavin from the mycelium. Inefficient cell lysis may, therefore, result in the retention of riboflavin in the vacuoles and thus reduced productivity. Therefore, we hypothesized that regulation of cell wall integrity (CWI) may be an important process to influence the

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

Strain	Genotype	Source
ALK4-1	*leu2, mpk1::GEN3/MPK1*	This study
ALK4-10	*leu2, mpk1::GEN3*	This study
ALK9-1	*leu2, mkk1::GEN3/MKK1*	This study
ALK9-10	*leu2, mkk1::GEN3*	This study
ALK14-1	*leu2, wsc1::GEN3/WSC1*	This study
ALK14-10	*leu2, wsc1::GEN3*	This study
ALK22-1	*leu2, swi4::GEN3/SWI4*	This study
ALK22-10	*leu2, swi4::GEN3*	This study
ALK27-1	*leu2, rlm1::GEN3/RLM1*	This study
ALK27-10	*leu2, rlm1::GEN3*	This study
ALK33-1	*leu2, pkc1::GEN3/PKC1*	This study
ALK33-10	*leu2, pkc1::GEN3*	This study
ALK39-1	*leu2, mid2::GEN3/MID2*	This study
ALK39-10	*leu2, mid2::GEN3*	This study
ALK40-1	*leu2, tus1::GEN3/TUS1*	This study
ALK40-10	*leu2, tus1::GEN3*	This study
AWL-20	*leu2, bck1::GEN3/BCK1*	This study
AWL-60	*leu2, bck1::GEN3*	This study
AWL-53	*leu2, swi6::GEN3/SWI6*	This study
AWL-55	*leu2, swi6::GEN3*	This study

All strains are derivatives of *Agleu2*.

amount of riboflavin produced by or released from the *A. gossypii* mycelium.

Here we present the functional analysis of ten genes of the *A. gossypii* CWI pathway. Prominent cell lysis phenotypes were found with several of the mutant strains, including *wsc1*, *pkc1* and the MAP kinase mutants. Cell lysis of MAP kinase mutants during stationary phase was, however, not beneficial for riboflavin recovery, presumably due to its premature onset. Interestingly, riboflavin yield in the *wsc1* mutant strain was increased. This opens a new avenue for genetic engineering of *A. gossypii* strains used for riboflavin production.

Materials and methods

Strains and media

A. gossypii strains were grown at 30 °C in liquid or on solid yeast extract-peptone-dextrose medium (AFM; 1% Bacto yeast extract, 1% caseine peptone, 2% dextrose). Mutant selection was promoted by the addition of G418/geneticin (200 μg/ml) or clonNAT (100 μg/ml). To obtain spores, strains were grown overnight in complete medium and then further incubated in minimal medium (1.7 g/l YNB w/o ammonium sulphate and w/o amino acids, 0.69 g/l CSM, 20 g/l glucose, 2 g/l asparagine and 1 g/l myo-inositol) for several days. The *A. gossypii* deletion strains generated in this study are listed in Table 1. Generally, for each gene deletion two independent transformants were generated. Some of the mutant phenotypes were complemented by transforming strains with freely replicating plasmids bearing the wild type gene (not shown). *Escherichia coli* strain DH5α served as host for plasmid propagations.

Gene deletions in A. gossypii

Electroporation of *A. gossypii* and the generation of gene deletion mutants using a PCR based approach was done as previously described (Wendland et al. 2000). Systematic ORF numbers of the *Ashbya* genes of interest and their corresponding *S. cerevisiae* homologs are listed in Table 2. Two independent heterokaryotic strains were initially isolated for each mutant. Spores of these mutants were germinated under selective conditions in AFM, and single homokaryotic mutants were isolated by microdissection using a Singer Instruments MSM 300 micromanipulator. Gene deletions were finally confirmed using diagnostic PCR (Walther and Wendland 2008). Primers were obtained from IDT (Integrated DNA Technologies, Leuven, Belgium) and are listed in Table 3.

Table 2
Comparison of CWI genes between *A. gossypii* and *S. cerevisiae*.

Gene	*Ashbya gossypii* systematic name	*Saccharomyces cerevisiae* systematic name	% identity
WSC1	ADL020W	YOR008C	35.2
WSC2/WSC3	AFR349W	YNL283C/YOL105C	30.4/34.4
WSC4	AFR191C	YHL028W	27.2
MID2	AEL302W	YLR332W	38.1
ROM1/ROM2	AFR585W	YGR070W YLR371W	56.5/59.5
TUS1	AER110W	YLR425W	44.5
RHO1	ABR182W ABR183W	YPR165W	75.6/85.0
PKC1	ACR191C	YBL105C	63.8
BCK1	AFR092W	YJL095W	50.2
MKK1/MKK2	ACR117W	YOR231W YPL140C	57.6/56.7
MPK1/KDX1	AER232C	YHR030C/YKL161C	78.0/52.7
SWI6	AFR690c	YLR182w	48.3
SWI4	AGL297c	YER111c	39.9
RLM1	AGR198c	YPL089c	36.2

Stress assays with A. gossypii strains

For cell stress assays, strains were pre-grown on regular AFM plates at 30 °C for three days, and subsequently re-spotted onto plates conferring various types of cell stresses. Plates were further incubated at 30 °C for an additional three days prior to photography. Strains were tested for different growth temperatures (AFM plates at 30 °C and 37 °C) and media conditions (AFM plates buffered with $CuSO_4$, 0.1 M $CaCl_2$ and 0.8 M mannitol).

Tip lysis/cytoplasmic leakage

Spores of *A. gossypii* mutants were spread onto selective AFM plates and incubated at 30 °C for 2–3 days. Pictures of colony edges were taken using a Zeiss Axiovert 200M inverse microscope equipped with a Roper Scientific/Photometrics CoolSNAP CCD camera and using the MetaMorph® image analysis software suite. In addition, cytoplasmic leakage was visualized using an overlay assay that was adapted from Kohli et al. (2008). For this, mutant strains were grown on AFM plates (3.2 cm diameter) at 30 °C for three days, until the colonies reached the outer rim of the plates. Subsequently, the plates were overlaid with a 1% agarose solution containing 0.05 M Tris/HCl buffer pH 9.5 and 10 mM BCIP (5-bromo-4-chloro-3′-indolyphosphate), an alkaline phosphatase substrate. Leakage of endogenous alkaline phosphatase drives a color reaction that was allowed to proceed for up to 1 h prior to photography.

Quantification of β-galactosidase expression in A. gossypii using an ONPG assay

To analyze the amount of recombinant β-galactosidase in culture supernatants, *A. gossypii* strains were transformed with either an empty vector or a plasmid containing the *lacZ* gene driven by the constitutive *S. cerevisiae TEF1* promoter. Transformants were pregrown over night in selective AFM and then diluted 1:10 in 50 ml of selective ½ AFM (0.5% caseine peptone and Bacto yeast extract, 2% glucose). After 3 days incubation at 30 °C, 50 μl media samples were taken and used in a standard liquid ONPG (ortho-nitrophenyl-β-D-galactopyranoside) assay (Rose and Botstein 1983).