PhD thesis
Lisa Wasserstrom

Molecular mechanisms regulating sporulation in the filamentous fungus *Ashbya gossypii*

Academic advisor: Steen Holmberg, Department of Biology, University of Copenhagen
Co-supervisor: Jürgen Wendland, Carlsberg Laboratory

Submitted: 06/08/14
Instituttav: Natur- og Biovedenskabelige Fakultet

Name of department: Department of Biology

Author: Lisa Wasserstrom

Titel: Molekylære mekanismer der regulerer sporulering i trådsvampen *Ashbya gossypii*

Title: Molecular mechanisms regulating sporulation in the filamentous fungus *Ashbya gossypii*

Academic advisor: Steen Holmberg, Jürgen Wendland

Submitted: 06.08.2014

Grade:

☐ Lorem ipsum in eternum

☐ Lorem ipsum in eternum
# Table of Contents

PREFACE .................................................................................................................. 5

SUMMARY ................................................................................................................. 6

RESUMÉ ...................................................................................................................... 7

LIST OF PAPERS ....................................................................................................... 8

ACKNOWLEDGEMENTS ............................................................................................ 9

INTRODUCTION ........................................................................................................ 12

AIMS OF THIS THESIS ............................................................................................ 13

MARIE CURIE INITIAL TRAINING NETWORK - ARIADNE .................................... 14

INTRODUCTION TO FUNGI .................................................................................... 15

* S. cerevisiae * ........................................................................................................... 15

* A. gossypii * ............................................................................................................ 16

Life cycle of * A. gossypii * ...................................................................................... 17

Genome of * A. gossypii * ......................................................................................... 17

THE BIOLOGY OF MATING ..................................................................................... 18

Mating type ............................................................................................................... 18

* MAT* locus in * S. cerevisiae * ............................................................................... 18

* MAT* locus in * A. gossypii * ................................................................................ 19

Positive and negative regulation of α-specific genes .............................................. 21

* A. gossypii * α-factor ............................................................................................. 21

Mating type switching in * S. cerevisiae * ............................................................... 22

The pheromone response pathway ....................................................................... 22

* S. cerevisiae * .......................................................................................................... 22

* A. gossypii* and other fungi .................................................................................. 23

Karyogamy ............................................................................................................... 24

MEIOSIS AND SPORULATION ................................................................................ 26

Regulation of meiosis in * S. cerevisiae * ................................................................. 26
Role of glucose in regulating sporulation ........................................................................28
  S. cerevisiae .................................................................................................................28
  A. gossypii ......................................................................................................................29
Meiotic recombination .....................................................................................................29
Meiotic recombination in A. gossypii ..............................................................................30
THE CELL WALL INTEGRITY PATHWAY .........................................................................31
  S. cerevisiae ..................................................................................................................31
  A. gossypii and other fungi ............................................................................................32
FINAL COMMENTS ..........................................................................................................32
SUMMARY OF PAPERS ....................................................................................................34
  Paper I ...........................................................................................................................34
  Paper II ..........................................................................................................................35
  Paper III .........................................................................................................................36
  Paper IV ........................................................................................................................37
  Paper V (not included in this thesis) ..............................................................................38
CONCLUSIONS AND FUTURE PERSPECTIVES ..........................................................39
APPENDIX I ......................................................................................................................51
Preface

This thesis “Molecular mechanisms regulating sporulation in the filamentous fungus *Ashbya gossypii*” presents the results of my PhD internship carried out at the Carlsberg Laboratory in Denmark. The project was supervised by Professor Dr. Jürgen Wendland at the Yeast Genetics Group, Carlsberg Laboratory and Professor Dr. Steen Holmberg at the Department of Biology, University of Copenhagen and funded by the European Union Marie Curie Initial Training Network, Ariadne.
Summary

Sporulation is a complex developmental program that fungi enter to ensure survival in unfavorable environmental conditions. Many fungal species are able to produce spores sexually through meiosis, which is beneficial since it introduces genetic variability into a population. The sexually reproducing ascomycete *Saccharomyces cerevisiae* has a well-defined sexual cycle, in which diploid cells can undergo meiosis and produce haploid spores in response to nutrient starvation. The diploid state is a requirement for meiosis and results from fusion of two haploid cells of the opposite mating type, which is regulated by the pheromone response pathway. Most ascomycetes have been reported to produce meiotic spores, however, a sexual cycle has not yet been identified in the filamentous fungus *Ashbya gossypii*. The main focus of my doctoral thesis has therefore been to understand the mechanisms behind sporulation in this fungus.

The lifecycle of *A. gossypii* starting with a haploid spore that matures into spore-containing mycelia can be completed without the need for a mating partner. Spores in *A. gossypii* are thought to be derived sexually like all other *Saccharomycetaceae* species, but the sexual cycle remains unidentified. In this thesis I provide a comprehensive functional analysis of genes important for sporulation in *A. gossypii*. Previous results, together with findings presented in this work show that the role of the pheromone response pathway in *A. gossypii* has been rewired to regulate sporulation negatively instead of controlling mating. In line with this, a mating partner might not be required since the multinucleate compartments could still enable nuclear fusion (karyogamy) and meiosis. The presence of karyogamy is supported by our results that deletion of the *A. gossypii* homologs Kar3 and Kar4, involved in karyogamy in *S. cerevisiae*, results in severely crippled sporulation.

This work also identifies the main regulators of sporulation in *A. gossypii*, namely *IME1, IME2, IME4, KAR4* and *NTD80*. Using large scale RNA sequencing data of these non-sporulating deletion strains we were able to identify 67 down-regulated genes, most of which were up-regulated in an oversporulating mutant, namely *ste12*. Interestingly, transcription of the main regulator of meiosis in yeast, *IME1*, is regulated by a significantly smaller promoter in *A. gossypii* and is independent of the transcription factors MsnP2 and Sok2. Furthermore, the role of Sok2 in *A. gossypii* has been rewired from a transcriptional repressor to an activator of sporulation. This is in contrast to *S. cerevisiae*, where Sok2 is a repressor of *IME1* transcription while MsnP2 and MsnP4 function as activators.

A prerequisite for meiosis is meiotic recombination that allows cross-over between homologous chromosomes. In *S. cerevisiae*, this requires double strand break (DSB) formation and subsequent repair via the components Spo11 and Dmc1. The work in this thesis show that the *A. gossypii* Spo11 and Dmc1 homologs are not required for sporulation, thus suggesting that other proteins generate DSBs in this fungus.

In summary, this work has led to better understanding of the components regulating sporulation in *A. gossypii* and their hierarchical organization.
Resumé

Sporedannelse er et komplext udviklingsmæssigt program, som svampe kan gennemgå for at sikre overlevelse under ugünstige miljøforhold. Mange arter af svampe kan producere seksuelle sporer gennem meiose, hvilket er fordelagtigt, fordi det indfører genetisk mangfoldighed i en population. Den seksuelt reproducerende ascomycet *Saccharomyces cerevisiae* har en velfungerer seksuel cyklus, hvor diploide celler kan undergå meiose og producere haploide sporer ved mangel på nærings. Diploide celler er afhængende for meiose og dannes gennem en fusion af to haploide celler af modsatte parring typer, hvilket reguleres af feromon response kaskaden. De fleste andre ascomyceter producerer også meiotiske sporer. Dog er en seksuel cyklus endnu ikke blevet identificeret i trädsvampen *Ashbya gossypii*. Hovedformålet med min afhandling har derfor været at forstå de mekanismer, som regulerer sporulering i denne svamp.


En forudsætning for meiose er meiotisk rekombination, der giver "cross-over" mellem homologe kromosomer. I *S. cerevisiae* kræver meiotisk rekombination et dobbeltskreng at brud (DSB) efterfulgt af reparation via komponent *Spo11* og Dmc1. Arbejdet i denne afhandling viser, at *A. gossypii* *Spo11* og Dmc1 homologerne ikke er påkrevet for sporulering, hvilket tyder på at andre proteiner genererer DSB’er i denne svamp.

Sammenfattende har dette arbejde givet en bedre forståelse af de komponenter, der regulerer sporulering i *A. gossypii* og deres hierarkiske organisation.
List of papers

I. Lisa Wasserstrom, Klaus B Lengeler, Andrea Walther and Jürgen Wendland (2014). Analysis of the PKA pathway in *Ashbya gossypii* reveals different modes of regulating sporulation that involves the positive elements Msn2 and Sok2. Manuscript.


Paper not included in this thesis


Paper II and III are reprinted with the permission of the publisher.
Acknowledgements

Without the people surrounding me both at work and at home, this thesis would not have been possible. My deepest thanks to all the people who contributed to the completion of this thesis, or in any other ways made my PhD life memorable.

First of all to my supervisor at Carlsberg Laboratory, Professor Jürgen Wendland for taking me under his wings and giving me the opportunity to work in the exciting field of fungal genetics. Thanks for always taking the time to discuss my project and generating new ideas and suggestions. Also, I am glad that I finally managed to teach you something with this thesis, feels like the best grade I could wish for.

Also, a special thanks to my academic supervisor, Professor Steen Holmberg for all your support during my work with this thesis.

Klaus, you got the “lucky” job to help me and Therese, the two lost PhD students, in getting started with the large Ashbya deletion project. With your patience and German determination we finally managed to generate the 80 mutants at last. Also a big thanks for always taking the time to answer my questions and for all the fun discussion we have had, both in the lab and the canteen.

Andrea, for welcoming me to the lab and helping out with all different research issues during my years at Carlsberg. You have a true talent for teaching and communication and your positive energy has inspired me many times.

Therese, min doktorandkollega och vän. Jag är väldig glad och tacksam att vi började doktorera samtidigt så vi kunde dela äventyren i Ariadne konsortiumet. Livet som doktorand var inte alltid lätt men du fanns där och var mitt stöd när jag behövde det. Också tack för pilen.

Davide, superman in the lab! Thanks for being supportive during the last stressful year of my PhD, especially with the preparation of the Fungibrain course. Keep up the good work and don’t ever stop using cartoons and fancy animations in your presentations ;).

Ana and Jevgenia, thanks for the great atmosphere you created both in your office and the canteen. I have enjoyed many talks with you and you definitively made life more fun and colorful at Carlsberg.

Claudia, I really enjoyed sharing lab bench with you this last year. Thanks for all the discussions about this and that and everything in between and also for the great trip we had to Madrid and Barcelona.

Klara, vi fick dela rum några månader här på slutet och du tillförde verkligen mycket till vår grupp med din energi och avslappnade inställning. Även om jag var inne i den intensiva slutfasen når du började så hoppas jag att jag hann lära dig någotting vetigt. Nu är det din tur att lära mig alla dina bryggeriprojekt och kanske jag kan "låna" lite olika jästsorter från dig.
Claes and Anna, thanks for your valuable help with the Danish summary, without you it would have been more Swedish than Danish.

Also, I would like to thank all the people at Carlsberg Laboratory for creating a stimulating research environment even in these hard times. Special thanks to Sabrina, Shakira, Mats and Anita for all the chats during train and bike rides and at the coffee machine.

Thanks to all the PIs, post docs and researchers in the Ariadne consortium that made it into such a success story. You all did a fantastic job in organizing the different courses including lectures and practical work and I am so grateful that I got the opportunity to be part of it. A special thanks to Doctor Elena Perez-Nadales for your endless work with the review article.

The Ariadnes: Betta, Clara, Ellie, Filomena, Katja, Mennat, Miriam, Pankaj, Sonia, Soode Therése and Vikram, What a journey we all have made, from unexperienced students to fellow researchers. It has been great fun to share research, laughter and all the PhD stress with you during these three years and I feel that I have made colleagues and friends forever. May the fungi be with you!


Anna, Cecilia, Charlotte och Åsa, jag är väldigt glad att vi håller kontakten i vårt lilla biolognätverk och det är alltid lika kul när vi ses och nördar ner oss i biologins värld.

Jill och Oskar, Johan, Mattias och Louise, Tina, Terese, Mathilda, ni är de finaste vänner som alltid finns där även om det ibland blir glöst mellan träffarna. Tack för Robinsontävlingar, otaliga fester, Köpenhamnsturer, körsäng, stughång och mycket annat. Utan er hade livet varit tomt!

Familjen Wasserstrom: Ewa och Adam, Maja och Daniel, Dorota, Ewa och Baba tack för att ni välkomnatt mig in i er familj. Särskilt tack till Ewa och Adam som hjälpt oss så mycket med Ellen. Utan er hade jag omöjligt kunnat slutföra min avhandling. Som tur väl är så har ni fätt betalt i flytande alkoholhaltig form.

Mamma och Farsan, tack för ert stöd under alla dessa år och att ni alltid trott på mig även om det har varit svårt att förstå vad det är jag sysslar med. Jag har alltid uppskattat er bådas beslutsamhet och glädje till ert arbete och jag är glad att jag ärvt delar av er energi. Mamma, du är stark och klok och tappar sällan fotfästet vilket jag beundrar mycket. Farsan, du var energisk som få och din livslädje smittade av sig till alla du mötte. Tack för att ni gjort det möjligt för mig att följa mina drömmar!


Introduction

Fungi inhabit a wide variety of environments including soil, water, plants, animals and humans. They can thrive in most climate zones on Earth as long as it is sufficiently moist and dark and organic material is available. In everyday life, most people find the encounter with fungi unpleasant because they unwedly grow and spoil food products such as bread, milk, cheese, meat and jam. However, fungi are very helpful and useful organisms if treated right. Many food products and beverages like beer, wine, bread, cheese and chocolate are derived from fungal fermentations. Fungi also have a large industrial impact due to their production of antibiotics, vitamins and enzymes that are used in food processing, biomass degradation and composting.

Life in these different habitats subject the fungus to stressors of various sorts that must be dealt with in order for the fungus to survive. Fungi therefore rely on their ability to sense and adapt to changes in the surrounding environment. This includes various stress factors like changes in temperature, pH, osmolarity and the availability of nutrients. These environmental changes are detected at the membrane of the cell by transmembrane sensors or receptors. The signal is conveyed, from the cell surface to the nucleus, to induce a transcriptional response. These highly complex signaling pathways allow the cell to quickly adapt to changing environments, which is of utter importance especially for a nonmotile specie (GUSTIN et al. 1998; FUCHS and MYLONAKIS 2009).

In response to nutrient limitations, fungi can enter the developmental pathway of meiosis and sporulation. As a result, spores are formed that allow the fungus to survive extended periods of time in unfavorable conditions. The dormancy of the spores is broken when nutrients become available again. Fungal spores can either be derived asexually, through mitotic division that generates clonal spores with the same genetic setup or, sexually through meiosis that results in a spore population with genetic variability. The outcome of sexual sporulation in higher fungal species is the formation of fruiting bodies that we are able to pick in the forest as mushrooms.

Sporulation through meiosis is well studied in the ascomycete S. cerevisiae. In this fungus, initiation of meiosis requires the presence of a non-fermentable carbon source and the absence of nitrogen and a fermentable carbon source. Meiosis and sporulation result in the formation of four spores that are packed into a sexual-spore bearing cell called ‘ascus’. A prerequisite for meiosis in S. cerevisiae is the diploid state that results from the fusion of two haploid cells of the opposite mating type (HERSKOWITZ 1988). In most other ascomycetes a sexual cycle has also been reported, however, with some exceptions including A. gossypii.

A. gossypii is a strictly filamentous fungus that is closely related to S. cerevisiae and 95% of the genes in A. gossypii share a homolog in S. cerevisiae (DIETRICH et al. 2004). It is homothallic and can thus complete its life cycle and generate haploid spores without the need for a mating partner. However, the intriguing question of whether these spores are derived from mitotic and meiotic divisions remains unanswered. Meiosis could occur without the need for mating since A. gossypii hyphae are multinucleate. Indeed many of the meiosis-specific components known from S. cerevisiae have homologs in A. gossypii, but some are also missing (WENDLAND et al. 2011; WASSERSTROM et al. 2013).

Understanding the developmental program resulting in sporulation in A. gossypii has been the main focus of my thesis. First I describe the complex pathways regulation mating, meiosis and sporulation in S. cerevisiae and compare to what is known in A. gossypii and other filamentous fungi. Then I present
my own work including the analysis of the *A. gossypii* homologs involved in, (i) the pheromone response pathway, (ii) karyogamy, (iii) initiation of meiosis, (iv) glucose sensing via the PKA pathway, and, (v) meiotic recombination. The results from this work are presented in paper I and II. In addition, a shorter study focusing on pH and sporulation is presented in paper IV. I also took part in the analysis of the cell wall integrity pathway in *A. gossypii* that facilitate cell wall remodeling and repair in response to cell wall stress. This work is presented in paper III.

Aims of this thesis

The main purpose of this thesis was to characterize the function of genes involved in sporulation and signaling transduction pathways in *A. gossypii* in order to elucidate their role in sporulation and riboflavin production. The specific aims were:

1. Based on a paradigm shift in the role of the pheromone response pathway in *A. gossypii* we aimed at understanding the molecular mechanisms that govern sporulation in this fungus.

2. To combine gene function studies and large scale RNA sequencing data into a model of how transcriptional regulators activate core sporulation-specific genes in *A. gossypii*.

3. To characterize the cAMP-dependent protein kinase A (PKA) pathway and identify key downstream effectors that promotes developmental decisions in switching vegetative growth to sporulation and riboflavin production in *A. gossypii*.

4. To investigate the function of the conserved *A. gossypii* MAP kinase signaling pathways regulated by pheromone and cell wall integrity in *S. cerevisiae*. 

13
Marie Curie Initial Training Network - Ariadne

My PhD project has been part of the European Union Marie Curie Initial Training Network, Ariadne, with the purpose to study signaling circuitry controlling fungal virulence. The ultimate goal of the consortium has been to identify and characterize conserved genes in the mitogen-activated protein kinase (MAPK) pathways that control fungal growth, development and reproduction and therefore play an important role in fungal virulence both in plants and humans (Rispaïl et al. 2009). A total of seven different fungal species have been studied in the consortium including the two major human fungal pathogens Aspergillus fumigatus and Candida albicans, the plant pathogens Fusarium oxysporum, Magnaporthe oryzae and Zymoseptoria tritici, as well as the two model organisms of filamentous and dimorphic fungi, A. gossypii and Ustilago maydis. My contributions to this consortium have involved studying the MAPK pathways in A. gossypii that in S. cerevisiae respond to: pheromone (Fus3 MAPK) and hypotonic shock (Mpk1 MAPK). The work in the Ariadne consortium resulted in a review in Fungal Genetics and Biology (paper V) that is not included in this thesis.
Introduction to fungi

The fungal kingdom comprises a large number of different species that thrive in a wide range of habitats. Fungi grow either yeast-like as individual cells, or as filaments, forming multicellular mycelia. Some fungi are dimorphic and able to switch from yeast-like to filamentous growth when exposed to certain extracellular signals (WENDLAND 2001).

Fungi are essential for decomposing organic matter that enables re-cycling of nutrients in the ecosystems and many plants rely on symbiotic fungi, so-called mycorrhizae that supply essential nutrients to the plant via the root system. In addition, fungi have a large industrial impact due to their production of secondary metabolites such as antibiotics, vitamins (e.g. riboflavin/vitamin B₂) and ethanol, but also various enzymes that are used in food processing, biomass degradation and composting (OLEPSKA-BEER et al. 2006). Other implications include the use of Trichoderma species as biological control agents. They protect plants from infection by soil-borne plant pathogens by competing for nutrients and space and by producing antibiotics and hydrolytic enzymes that kill the pathogenic fungi (HERMOSA et al. 2013). Another fungus, Fusarium venenatum, is used in Quorn™ to provide a meat free protein source (“mycoprotein”) for humans low in cholesterol and fat (WIEBE 2002). Fungi are also serious pathogens both on plants and in animals and humans, especially in immunocompromised individuals. Because of their close relationship to animals, fungi-associated infections can be hard to treat, especially since they form resilient biofilms on medical implantations (DEAN et al. 2012).

*S. cerevisiae*

*S. cerevisiae* is a unicellular yeast that has been used throughout history in baking, winemaking, brewing and nowadays also for bioethanol production. *S. cerevisiae* has the unique ability to ferment sugars into ethanol both under aerobic and anaerobic conditions. In nature it colonizes on the skin of ripening fruits, where it degrades the freely available mono- and oligosaccharides (DASHKO et al. 2014). *S. cerevisiae* has become a eukaryotic model organism due to its compact genome and the fact that it can easily be cultured and transformed, which allows for genetic modifications by homologous recombination. *S. cerevisiae* has a well-defined sexual cycle and therefore produces meiotic spores, which has been used extensively to understand the mechanisms behind sexual reproduction, recombination and DNA repair (BOTSTEIN and FINK 2011). It divides by budding, where a daughter cell made of entirely new cell surface material emerges from the mother cell and is separated by cytokinesis.

Even though it is unicellular, *S. cerevisiae* exist in three distinguishable cell types that all can undergo mitosis. The two specialized haploid cell types are of mating type a or α. In response to mating pheromone of the opposite cell type, α and a cells form a cell projection that enables them to mate and fuse with each other to form diploid a/α cells containing one nucleus. This diploid a/α cell is not able to mate but can undergo meiosis and sporulation to produce four haploid spores: two a and two α spores (Figure 1) (HERSKOWITZ 1988; Bi and PARK 2012).

One interesting feature with *S. cerevisiae* is that haploid cells can switch their mating type. Cells that originally carried the same *MAT* locus information, e.g. *MATa*, can switch to gain an active *MATα* locus instead, enabling them to mate with each other (HABER 1998). Such self-fertile cells that are able to generate diploid progeny from a single haploid cell are referred to as homothallic. On the contrary, strains that require mating of two cells derived from separate spores with opposite mating-type to
produce diploid progeny are referred to as heterothallic or “self-sterile”. In nature, both homothallic and heterothallic strains of *S. cerevisiae* are found and they differ only in one gene, denoted HO (homothallism), which is essential for mating-type switching. Homothallic strains have a functional HO allele while heterothallic strains have a defective copy (HERSKOWITZ 1988).

The complete genome sequence of *S. cerevisiae* published in 1996 contains 12 million base pairs that encode 5800 proteins dispersed on 16 chromosomes. It was the first eukaryotic genome ever sequenced and resulted from a 7 year collaborative project involving 600 scientists and 100 laboratories (MEWES et al. 1997; ZAGULSKI et al. 1998). Shortly after the genome was published, the *Saccharomyces* gene deletion project was initiated that generated an almost complete set of open reading frame deletion strains (WINZELER et al. 1999; GIAEVER et al. 2002; BOTSTEIN and FINK 2011). This was possible due to the efficient system for homologous recombination that allows integration of linear DNA with homology of less than 50 bp, i.e. PCR based gene targeting (LORENZ et al. 1995).

![Diagram of the meiotic life cycle of *S. cerevisiae*](image)

**Figure 1. The meiotic life cycle of *S. cerevisiae*.** The three cell types of *S. cerevisiae*, α, a, and α/a, can undergo mitotic cell division by budding (1). In response to mating pheromone of the opposite mating type, haploid a and α cells form cell projections, so-called shmoos (2), which enables them to fuse and form a diploid α/a zygote (3). The diploid a/α cell (4) is not able to mate but will go through meiosis and sporulation when the environmental conditions are appropriate and form an ascus containing four haploid spores (5). The spores are released from the ascus and after germination they start growing vegetatively again (6) (HERSKOWITZ 1988; Bt and PARK 2012).

**A. gossypii**

*A. gossypii* is a filamentous ascomycete that belongs to the genus *Eremothecium*. It was first discovered as a plant pathogen causing stigmateomycosis in cotton plants and was isolated from infected cotton (*Gossypium hirsutum*) by Ashby and Novell already in 1926. In order to spread the spores and infect the plant, *A. gossypii* relies on hemipteran insects, i.e. cotton strainers (ASHBY and NOWELL 1926). Since the emergence of insecticides, the disease is easily controlled and the fungus is not considered to be a devastating plant pathogen (WENDELAND and WALTHER 2005).

*A. gossypii* is as an overproducer of riboflavin (vitamin B₂), which is responsible for its yellow color (WICKERHAM et al. 1946). Riboflavin is a precursor of the flavonogenic coenzymes FMN and FAD that
are essential components of oxidoreductases in the electron transport chain. Riboflavin has a commercial value and is used in the food industry as an additive and colorant. Therefore, riboflavin biosynthesis dominated the Ashbya research for many years and has resulted in today’s use of the fungus to produce riboflavin on an industrial scale (STAHMANN et al. 2000).

**Life cycle of A. gossypii**

The life cycle of *A. gossypii* starts with germination of the needle shaped haploid spore that grows to form multinucleated hyphae containing compartments separated by chitin-rich septa (Figure 2). The germination of the spore starts with a short period of isotropic growth to generate a spherical germ cell. Upon switching to polarized growth, the first germ tube will emerge after about 8 hours. Next, a germ tube form at the opposite side generating a bipolar germination pattern. As the mycelium continues to grow by lateral branching, a compact juvenile mycelium forms. About 20 hours post-germination the hyphae mature, which results in a switch to dichotomous branching (tip splitting) that generates the characteristic Y-shaped hyphae (WENDLAND and WALther 2005). The tip splitting speeds up growth from 6-10 µm/h to 200 µm/h (KNECHTLE et al. 2003). The life cycle is completed when the older parts of the mycelium have produced new spores in response to nutrient limitations. This causes the hyphal segments between the septa to enlarge and form single-celled sporangia that contain the spores (WENDLAND and WALther 2005). *A. gossypii* can complete its lifecycle starting from a haploid spore that matures into spore-containing mycelia without the need for a mating partner and may therefore be defined as homothallic (WENDLAND et al. 2011). However, meiosis has not yet been observed (WENDLAND et al. 2011; WASSERSTROM et al. 2013).

Figure 2. Life cycle of *A. gossypii*. The life cycle starts with germination of the haploid spore by a short period of isotropic growth to form the germ cell (1). The first germ tube will emerge after ca 8 hours (2) and soon after a second germ tube forms, generating a bipolar germination pattern (3). The mycelia continue to grow by lateral branching forming a juvenile mycelia (4). About 20 hours post-germination the hyphae start to grow by dichotomous branching forming the characteristic Y-shaped hyphae (5). The life cycle is completed when the hyphal compartments swell to form sporangia and each nucleus is packed into a haploid spore (6). The life cycle ends when the spore-containing sporangia lyse and the spores are released (7) (WENDLAND and WALther 2005).

**Genome of A. gossypii**

Despite its filamentous life style, *A. gossypii* is more closely related to yeast than to other filamentous fungi. The A.g genome was published in 2004 and revealed a compact genome of 9.2 million base pairs that harbors 4718 protein-coding genes distributed over 7 chromosomes. More than 90 % of the
genes share both homology and synteny with S. cerevisiae (Dietrich et al. 2004). A. gossypii and S. cerevisiae originate from a common ancestor that carried eight chromosomes and about 4700 genes. The two species diverged somewhat hundred million years ago, and S. cerevisiae went through a whole genome duplication (WGD) event to generate the 32 chromosomes it harbors today. From the WGD, S. cerevisiae has only kept 500 of the gene pairs in duplicate copies. A. gossypii on the other hand has reduced the number of chromosomes to seven by breakage of one chromosome at its centromere followed by fusion of these two arms to the telomeric ends of two different chromosomes. This centromere breakage is a unique event so far among the Saccharomyces species (Gordon et al. 2011).

Recently, two other Eremothecium species have been sequenced, namely E. cymbalariae and E. coryli. E. cymbalariae harbors 4712 genes distributed on 8 chromosomes (Wendland and Walthner 2011) while E. coryli harbors 4682 genes on 6 chromosomes (Wendland and Walthner 2014). This chromosome reduction from 8 to 6 in E. coryli has resulted from two telomere-to-telomere fusions.

The fact that A. gossypii is closely related to S. cerevisiae but display such a different life style made it an interesting model organism for studying filamentous growth (see phylogenetic tree in Figure 3). In addition, genetic manipulations are made easy in Ashbya due to the efficient system for homologous recombination that allows PCR based gene targeting just like in S. cerevisiae. Other filamentous fungi have high frequency of ectopic integration at nonhomologous positions that makes genetic manipulations that much harder (Wendland et al. 2000; Wendland 2003).

Figure 3. Phylogenetic tree of the Saccharomyces clade. The arrow shows the whole genome duplication event (WGD) (Butler et al. 2009).

The biology of mating

Mating type

MAT locus in S. cerevisiae

As mentioned earlier, the two different haploid cell types in S. cerevisiae, a and α, are able to mate and form a diploid a/α cell that can undergo meiosis and generate spores. This sexual mating is controlled by genes encoded at the MAT locus on chromosome 3 that contains alleles of either MATa or MATα. In addition, two silent copies of MATa and MATα are present at the telomeric ends of chromosome 3, denoted HMLα and HMRα, i.e. Hidden MAT Left /Right (Figure 4). Cells expressing genes of MATa mate as a cells while cells expressing MATα mate as α cells (Haber 1998).
**S. cerevisiae**

**Figure 4. MAT locus in S. cerevisiae.** The active MAT locus on chromosome 3 harbor either α1 or α1/α2 information. In addition, a 24 bp recognition site for the Ho endonuclease is present that enables mating-type switching. Two silent MAT loci denoted HMLα and HMRα are present at the telomeric ends (HABER 1998).

MATαα encodes two proteins, α1 and α2. α1 forms a heterodimer together with Mcm1 that activates α-specific genes (αsg). This includes genes encoding the a-pheromone receptor Ste3 and the α-mating pheromone (MFα1 and MFα2). On the contrary, α2 acts with Mcm1 and also Tup1 and Ssn6 to form a repressor that inhibits expression of α-specific genes (αsg) including genes encoding the α-mating pheromone (MFα1 and MFα2) and Ste2, the a-pheromone receptor (JENSEN et al. 1983; GOUTTE and JOHNSON 1988; STRATHEARN et al. 1988; LI et al. 1998). Since the MATα locus is required to both activate α-specific genes and inhibit a-specific genes, disruption of the locus result in cells of a-mating type (STRATHEARN et al. 1981).

MATαα encode one protein, α1 that is not required for a-specific mating, however, α1 forms an essential co-repressor with α2 that represses haploid specific genes (hsg) including the meiotic repressors RME1 and RME2, to allow meiosis in diploid cells (Figure 5) (LI et al. 1995; HABER 1998; HONGAY et al. 2006).

**Figure 5. Regulation of mating-type specific genes in S. cerevisiae.** In a-cells, a-specific genes (αsg) and haploid specific genes (hsg) are activated by default since the α1 protein is not required for a-specific mating. In α-cells, a-specific genes (αsg) are activated by α1 and Mcm1 while αsg is inactivated by α2 and Mcm1. In diploid a/α cells, expression of αsg is inhibited by α2 and Mcm1, and α1 together with α2 forms a heterodimer that inhibits transcription of hsgs and α1. See text for more details.

**MAT locus in A. gossypii**

The genome of A. gossypii ATCC10895 harbors four identical MATα loci present on chromosomes 4, 5 and 6. In contrast to yeast, the MATα locus of A. gossypii encodes two proteins, α1 and α2, similarly to C. albicans, Kluyveromyces lactis and the common yeast ancestor (JOHNSON 2003; TSONG et al. 2003; TSONG et al. 2006; WENDLAND and WALThER 2011). The two loci on chromosomes 4 and 5, MAT2 and MAT3, and the MAT4 locus on chromosome 6 are located close to the telomeric ends, most likely resembling the silent telomeric MAT loci, HMRα found in S. cerevisiae. The presumptive active MAT1 locus is present on chromosome 6. Interestingly, another Ashbya isolate named A. aceri was recently sequenced and shows 99.9 % genome identity with A. gossypii, however, A. aceri contains α1/α2 information at the MAT4 locus. The gene order of the surrounding genes at the MAT4 locus is conserved between the species. It is therefore likely that MAT4 of A. gossypii originally carried α1/α2 genes but in a gene conversion event it was replaced by the MAT2 locus on chromosome 4. In addition,
PCR assays show that 5 other wild isolates of *A. gossypii* encode MATα and MATα sequences (Dietrich et al. 2013). The close relative to *Ashbya, E. cymbalariae* has three mating type loci on chromosome 1 similarly to what is found on chromosome 3 in *S. cerevisiae* and *K. lactis* (Wendland and Walther 2011). The MAT locus of *C. albicans* harbors either a1/a2 or a1/a2 information. The locus also contains three additional genes, *PAP, OBP* and *PIK*, which encode a poly (A) polymerase protein, an oxysterol binding protein and a phosphoinositide kinase protein, respectively (Bennett and Johnson 2005).

A schematic picture of the MAT locus organization in the different ascomycetes *S. cerevisiae, E. cymbalariae, K. lactis, A. gossypii* and *C. albicans* is presented in Figure 6.

---

**Figure 6.** MAT locus organization in different ascomycetes. *S. cerevisiae* has two silent telomeric MAT loci, HMLα and HMLα, and one active MAT locus on chromosome 3 that harbor either a1 or a1/a2 information. The active MAT locus of *S. cerevisiae* also contains a 24 bp recognition site for the Ho endonuclease that enables mating type switching (Haber 1998). *E. cymbalariae* and *K. lactis* show a similar MAT organization on chromosome 1 and 3, respectively (Wendland and Walther 2011). *A. gossypii* harbors 4 identical MATα loci on chromosome 4, 5 and 6. MAT1 on chromosome 6 is the presumptive active locus while MAT2, MAT3 and MAT4 are all telomeric and most likely remnants of the silent HMLα cassette of *S. cerevisiae*. The MAT4 locus of the close relative, *A. aceri* contains a1 and a2 information (Dietrich et al. 2013). The *C. albicans* mating locus either contains a1/a2 or a1/a2 information. In addition, the locus in *C. albicans* harbors three additional pairs of genes, *PAP, OBP* and *PIK* that are not present in most other fungi (Bennett and Johnson 2005).
Positive and negative regulation of a-specific genes
In *C. albicans*, Mata2 positively regulates asg together with Mcm1 (TSONG et al. 2003) while in *S. cerevisiae* the gene encoding the a2 protein has been lost and instead asg are regulated negatively by α2 and Mcm1 (BUTLER et al. 2004). This evolutionary transition from positive to negative regulation of the a-specific genes among the ascomycetes is described in Figure 7. The positive regulation via a2 was present in the yeast ancestor and is therefore referred to as the ‘ancestral state’. The α2-repression was gained while the ancestral form of a-specific regulation was still present and therefore some species have both modes of regulation, here called the ‘hybrid state’ (TSONG et al. 2006). The ‘derived state’ refers to *S. cerevisiae* that has lost the a2 activation and only kept the α2 repression. In *A. gossypii*, the α2 protein is functional and can repress expression of both *S. cerevisiae* and *A. gossypii* asg. In addition, the presence of a functional a2 protein suggests that *A. gossypii* might have retained a hybrid regulatory state (BAKER et al. 2012).

![Figure 7. Evolutionary transition of a-specific gene regulation.](image)

In the ancestral state, represented by *C. albicans* and the yeast ancestor, asg are regulated positively via a2/Mcm1. During evolution, a negative mode of regulating asg via α2/Mcm1 repression was gained while the positive mode was still present, referred to as the hybrid state. In *S. cerevisiae*, the positive regulation has been lost and only the α2/Mcm1 repression remains, referred to as the derived state (TSONG et al. 2003; TSONG et al. 2006; BAKER et al. 2012).

**A. gossypii α-factor**
*A. gossypii* was found to have two α-factor encoding genes, *AFL062W* and *AAR163W*, homologous to the *S. cerevisiae* MFα1 and MFα2, respectively. AgMFα1 does not encode a functional peptide while AgMFα2 encodes a precursor protein that harbors one copy of the mature pheromone. This is in contrast to other yeasts where MFα1 and MFα2 contain several copies of mature pheromones. Interestingly, the *A. gossypii* α-factor encoded by AgMFα2 together with the α-pheromone receptor AgSte2 are functional in transmitting the signal when heterologously expressed in *S. cerevisiae* (WENDLAND et al. 2011).
Mating type switching in *S. cerevisiae*

Haploid *S. cerevisiae* cells can change their mating-type from one state to the other. This allows mating of cells that originally carried the same *MAT* locus information, e.g. *MATa*, but after mating-type switching one cell has gained an active *MATα* locus instead. This process of self-mating allows a pseudo-homothallic haploid strain to mate into the diploid state. In nature it is very favorable to be in the diploid state since a diploid cell is able to undergo meiosis and sporulation under nutrient limiting conditions. Also, advantageous for diploid cells is that they can correct deleterious mutations in one chromosomal copy by homologous recombination (Haber 1998).

Mating type switching is a highly site-specific recombination event that replaces the active *MAT* locus with the telomeric *MAT* of the opposite mating type. This is initiated by the Ho endonuclease through a double strand break (DSB) in the 24 bp recognition site present in the active *MAT* locus. Once Ho has cut the *MAT* locus, exonucleases degrade the DNA on both sides of the DSB, generating 3′-ended tails. To repair the *MAT* locus, new sequences are copied from either *HMLα* or *HMRα* in a strictly controlled gene conversion process. There are mechanisms to ensure that e.g. *MATa* will recombine with *HMLα* and not *HMRα* in order for a mating type switch to occur (Haber 2012). Expression of the *HO* gene is tightly regulated to make sure that a DSB is only induced in haploid mother cells under the G1 stage of the cell cycle (Nasmyth and Shore 1987). Neither genomes of *A. gossypii* nor *E. cumbalariae* contain a homolog to *HO* (Wendland and Walther 2011).

The pheromone response pathway

*S. cerevisiae*

In *S. cerevisiae*, the mating response that enables fusion of two haploid cells is controlled by the pheromone MAPK pathway. To describe it shortly, binding of pheromone to the G-protein coupled receptor transmits the signal via the MAP kinase cascade to activate genes regulating G1-arrest, shmoo formation and cell fusion. A schematic diagram of the pathway is presented in Figure 8.

The pathway is activated when pheromone of the opposite mating type binds to the G-protein coupled pheromone receptors Ste2 or Ste3. This causes a GDP/GTP exchange in the Gα-subunit Gpa1 that releases the membrane bound Gβγ heterodimer Ste4/Ste18. Gβγ associates with the scaffold protein Ste5 and the PAK kinase Ste20 and localizes them to the membrane (Pryciak and Huntress 1998; Good et al. 2009). The binding of Ste20 to the membrane and its subsequent activation requires the Rho-like G-protein Cdc42, the guanine nucleotide exchange factor Cdc24 and Bem1. At the membrane, Ste5 facilitates phosphorylation of Ste11, via Ste20, by acting as a scaffold that organizes the MAPK cascade proteins Ste11 (MAPKKK), Ste7 (MAPKK) and Fus3 (MAPK) and enables the signal transmission between them. The main targets of the Fus3 MAPK cascade are the transcription factors Far1 and Ste12 that regulate about 200 mating-specific genes involved in G1 arrest, shmoo formation and cell fusion. Ste12 activates pheromone responsive genes by binding as a homodimer to promoters containing the consensus TGAAACA called the pheromone response element (PRE). It also activates a-specific genes together with the MADS box protein Mcm1 (Bardwell 2005).

The same MAPK module, but with Kss1 replacing Fus3, is regulating pseudohyphal and invasive growth in response to nutrient limitation. Many of the components are shared between the Fus3 and Kss1 MAPK pathways with some exceptions. Msb2 and Sho1 are the membrane bound sensors that transmit the starvation signal in the Kss1 pathway. The scaffold protein Ste5 is only required for
activation of Fus3 and not Kss1. Activated Kss1 transmits the filamentation response to the transcription factor Tec1 indirectly via phosphorylation of the TEC1 transcriptional repressor Dig2 (Chou et al. 2006). Tec1 is a transcription factor of the TEA/ATTS family that recognizes and binds to the sequence CATTC(C/T) temed TEA consensus sequence elements, TCS. In order to achieve signal specificity, the filamentation induced genes are activated by cooperative binding of Ste12 and Tec1 to enhancer elements called filamentation and invasion response elements (FREs) that contain a PRE close to a TCS site (Madhani and Fink 1997). Also, Tec1 activity is prevented during the mating response by Fus3-mediated phosphorylation that marks the protein for degradation (Chou et al. 2006). Both Ste12 and Tec1 are inhibited in unstimulated haploid cells by Dig1 and Dig2 (Chen and Thörner 2007). Deletion of DIG1 and DIG2 in diploid α/α cells leads to constitutive expression of STE12 and a-specific genes. These diploid cells are able to mate as a-cells and therefore prohibited from sporulating (Gelli 2002).

![Figure 8. Diagram of the MAP kinase pathways transducing the pheromone response and starvation/filamentous growth response in S. cerevisiae.](image)

The pheromone signal is transmitted through the plasma membrane via the pheromone receptor Ste2 or Ste3 to induce disassociation of the G-protein. The membrane bound βγ subunits recruit Ste20 and the scaffold protein Ste5 to the membrane that facilitates phosphorylation and activation of the MAP kinase cascade. This leads to a transcriptional activation of Ste12 that acts as a homodimer to activate mating genes containing a PRE element. The starvation response is transduced by the sensors Msb2 and Sho1 via the MAP kinase cascade to the transcription factors Tec1 and Ste12 that induce transcription of filamentation genes by cooperatively binding TCS and PRE elements. See text for more details.

### A. gossypii and other fungi

In A. gossypii, all the components of the Fus3/Kss1 MAPK are present, including Ste5 and Dig1 that are not well conserved in other filamentous fungi (Rispail et al. 2009). However, the function of the cascade does not seem to involve mating since a strain deleted for both genes encoding the pheromone receptors, Ste2 and Ste3, is still able to sporulate. Furthermore, deletion of STE12 results in a hypersporulation phenotype in A. gossypii, which is not seen in yeast or other filamentous fungi. Interestingly, deletion of TEC1 results in a similar hypersporulation phenotype, which indicate that Tec1 and Ste12 might regulate sporulation in a cooperative manner in A. gossypii (Wendland et al. 2011).
The Fus3/Kss1 MAPK cascade is highly conserved in eukaryotes and has an important role in regulating virulence in pathogenic fungi. The **FUS3** ortholog **PMK1** is essential for pathogenicity in the rice blast fungus *M. oryzae*, pmk1 mutants are unable to penetrate the host and grow invasively due to lack of the infectious structures called appressoria (Zhao et al. 2007). The pmk1 mutant of *Magnaporthe grisea* also fails to infect rice tissue when inoculated directly in wound sites (Xu and Hamer 1996). Similarly, pathogenicity is completely lost when the **PMK1** orthologue **FMK1** is deleted in the non-appressorium-forming soil-borne vascular wilt fungus *F. oxysporum* (Di Pietro et al. 2001). The ortholog of the main transcription factor of the cascade, Ste12, is also required for mating in filamentous fungi. Deletion of **STE12** results in defective sexual cycles in several fungal species including *Aspergillus nidulans*, *Botrytis cinerea*, *C. albicans*, *F. oxysporum* and *Neurospora crassa* (Liu et al. 1994; Vallim et al. 2000; Li et al. 2005; Rispaïl and Di Pietro 2009; Rispaïl and Di Pietro 2010; Schamber et al. 2010). Common for Ste12 in filamentous fungi is that the C-terminal part of the protein contains a C2H2 zinc finger domain that is not present in *S. cerevisiae* or *A. gossypii*. The role of this domain is not known since removal has no effect on DNA binding in vitro but is required for a functional Ste12 in vivo as shown in *M. oryzae* (Park et al. 2004; Wendland et al. 2011).

**Karyogamy**

Activation of the pheromone MAPK pathway results in the fusion of two haploid cells to one diploid cell. After cell fusion the two nuclei need to migrate towards each other and fuse to form one single diploid nucleus, a process referred to as karyogamy. Prior to cell fusion, microtubules (MTs) originating from the shmoo tip are anchored at the MT organizing center, the so-called spindle pole body (SPB) of the nucleus to orient it towards the direction of the cell fusion. This prepares the nuclei for karyogamy, which is initiated directly after cell fusion. A schematic picture is presented in Figure 9.

During karyogamy, the two nuclei migrate toward each other on MTs in a process referred to as ‘nuclear congression’ that requires Kar1, Kar3, Kar4 and Kar9. Kar1 is essential for SPB duplication in mitosis and localizes other proteins to the SPB during karyogamy. **KAR3** encodes a minus end-directed kinesin-14 motor that anchors to the SPB and uses MTs to pull the two nuclei together with minus end-force (Meluh and Rose 1990; Gibeaux et al. 2013). **KAR3** expression is regulated by the transcription factor Kar4 that is activated by the pheromone response pathway. Kar4 also controls transcription of **CYK1** that encodes the associated light chain protein of Kar3 (Kurihara et al. 1996; Gammie et al. 1999; Lahav et al. 2007). Kar9 interacts with Myo5 to direct the nucleus toward the shmoo tip by moving the microtubules along polarized actin cables (Gibeaux and Knop 2013).

When the nuclei are positioned close enough, the nuclear membranes will fuse to produce a diploid nucleus. This process requires Kar2, Kar5, Kar7 and Kar8. Kar5 together with Prm3 are essential for nuclear fusion of the other membrane while Kar2 and Kar8 carry out the inner membrane fusion (Melloy et al. 2009). Kar7 is required for synthesis and possible also for the stability of Kar5 (Brizzio et al. 1999).
Figure 9. Schematic picture of mating, cell fusion and karyogamy in *S. cerevisiae*. Haploid cells respond to pheromone (α-factor or α-factor) of the opposite mating type by forming a cell projection (shmoo) that allows them to fuse and produce a diploid cell. Prior to cell fusion, the nucleus is attached to the shmoo tip by microtubules (black lines) that bind to the spindle pole body (red square). Once the cells have fused the nuclei get pulled in close proximity in a process called nuclear congression that requires Kar1, Kar3, Kar4 and Kar9. Next, the outer and inner membranes of the two nuclei fuse to produce one single diploid nucleus. The nuclear fusion involves the proteins Kar2, Kar5, Kar7 and Kar8 (ROSE 1996; GIBEAUX and KNOP 2013).

Kar3 has a special important role during nuclear congression as it is the motor protein that moves the nuclei towards each other along the MTs (GIBEAUX et al. 2013). Kar3 is also important for mitosis and has functions in different MT-required events during vegetative growth (SHANKS et al. 2001). Nuclear congression is initiated by the pheromone pathway through activation of Kar1 that interacts with Spc72 and relocates the MTs bound to Spc72 from the SPB to an extension of the SPB referred to as the half bridge. Subsequently, Kar3 binds to Spc72 together with the kinesin-related protein Cik1. After cell fusion, the microtubules from the mating partner protrude into the cytoplasm and as they reach the opposite spindle pole body and interact with Spc72-achedored Kar3, the nuclear congression starts (Figure 10) (GIBEAUX and KNOP 2013).

Figure 10. Nuclear congression during karyogamy. Upon pheromone binding, the MAPK cascade consisting of Ste11, Ste7 and Fus3 becomes phosphorylated, which results in Fus3-dependent activation of transcription factors regulating cell cycle arrest, cell and nuclear fusion. This activates Kar1 and Kar3 that are important for nuclear congression during karyogamy. Kar1 interacts with Spc72 and relocates the microtubules (MTs) from the spindle pole body (SPB) to the half-bridge that is membrane anchored to Kar1. The kinesin-like motor protein Kar3 together with Cik1 binds to Spc72 and as MTs from the mating cell reach the opposite SPB, Kar3/Cik1 pulls the two nuclei together by minus-end directed force (GIBEAUX and KNOP 2013).

Expression of KAR3 and CIK1 is dependent on the Kar4 transcription factor. The Kar4 protein exists in two forms, a constitutive, longer form (335 residues) that is mostly expressed during vegetative
growth and a shorter form (305 residues) that is predominantly transcribed during mating. Transcription of the shorter mating-induced KAR4 is activated by Ste12, and together they initiate expression of KAR3 and CIKI in response to pheromone (GAMMIE et al. 1999). In A. gossypii KAR4 this second in-frame transcriptional start codon, present 90 bp downstream from the first ATG in ScKAR4, does not exist. However, AgKAR4 expression appears to be regulated by Ste12 and Tec1 since both PREs and TCSs are found in the promoter (Table 1). In paper II we show that both Kar3 and Kar4 indeed are involved in regulating sporulation in A. gossypii since deletion results in mutants with defects in sporulation.

Table 1. Binding sites in the KAR4-promoter. The presence of Ste12 binding pheromone response elements (PRE) and Tec1 binding sites (TCS) in the KAR4-promoter of S. cerevisiae and A. gossypii.

<table>
<thead>
<tr>
<th>KAR4-promoter Position</th>
<th>Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
</tr>
<tr>
<td>Ste12 binding site</td>
<td>-93</td>
</tr>
<tr>
<td>(PRE)</td>
<td>-115</td>
</tr>
<tr>
<td></td>
<td>-138</td>
</tr>
<tr>
<td></td>
<td>-187</td>
</tr>
<tr>
<td><strong>A. gossypii</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-127</td>
</tr>
<tr>
<td></td>
<td>-270</td>
</tr>
<tr>
<td>Tec1 binding site</td>
<td>-134</td>
</tr>
</tbody>
</table>

Meiosis and sporulation

Regulation of meiosis in S. cerevisiae

In the diploid state, S. cerevisiae can undergo meiosis and form haploid progeny in response to nutrient limitations. This switch between mitosis and meiosis is tightly controlled by genetic and nutritional signals including (i) cell type (haploid or diploid), (ii) nutrient availability and (iii) respiration. First, only diploid cells are able to sporulate due to the presence of the α1/α2 heterodimer. Second, starvation for nitrogen and glucose is required for G1 arrest and inactivation of the PKA pathway, respectively. Finally, a non-fermentable carbon source such as acetate or ethanol is necessary to force the cell to respire. Respiration is important both as a signal to induce sporulation by increasing the pH and to provide energy for the developmental sporulation program (PIEKARSKA et al. 2010). The role of pH in regulating sporulation in A. gossypii is studied in paper IV. All these signals converge to transcriptionally regulate a specific set of genes essential for the initiation of meiosis, namely IME1, IME2, IME4, NDT80 and UME6 (Figure 11).

Ime1 is the master regulator of meiosis and activates the early meiotic genes (EMG) (KASSIR et al. 1988) that are important for pre-meiotic DNA replication, meiosis-specific chromosome remodeling and homologous recombination. Ime1 itself is not a DNA binding protein, but is recruited to the URS1 upstream regulatory sequence elements (TAGCCGCGCG) of early gene promoters by Ume6. During vegetative growth, Ume6 acts as a repressor of EMG by recruiting the Sin3 co-repressor and Rpd3 histone deacetylase complex to their promoters. In response to nitrogen starvation, Ume6 is
turned into an activator of EMG by recruiting Ime1 to early gene promoters. To allow activation of EMG, Ume6 is degraded by ubiquitin-dependent protein degradation (MALLORY et al. 2007; VAN WERVEN and AMON 2011).

IME1 transcription is controlled by an unusually large promoter, > 2.1 kb, which contains both positive and negative regulatory elements called upstream controlling sequences, UCS1-4 (SAGEE et al. 1998). UCS1 prevents IME1 transcription when nitrogen is available, while UCS2 activates transcription in response to a non-fermentable carbon source. In addition, a glucose signal is transmitted to UCS2 via the cAMP-dependent protein kinase A (PKA) pathway to block IME1 expression. UCS3 and UCS4 are regulated by the repressor Rme1 in haploid, but not diploid cells since the a1/a2 heterodimer inhibits RME1 transcription. (SAGEE et al. 1998). Rme1 acts as a repressor by inducing the expression of an intergenic noncoding RNA, IRT1 that covers the majority of the IME1 promoter and thereby blocks transcription (VAN WERVEN et al. 2012).

Transcriptional induction of IME1 in diploid cells is also dependent on Ime4 that is thought to be regulated by nutritional signals. Ime4 is a N6-methyladenosine mRNA methyltransferase that methylates mRNA of IME1, IME2 and IME4 itself (SHAH and CLANCY 1992; BODI et al. 2010). IME4 expression is controlled by an antisense transcript called RME2 that represses transcription in haploid cells. RME2 expression is inhibited in diploid cells by the a1/a2 heterodimer (MITCHELL and HERSKOWITZ 1986; HONGAY et al. 2006).

![Mating-type control and Nutritional control](image)

**Figure 11. Positive and negative regulation of IME1 transcription in S. cerevisiae.** The 2.1 kb IME1-promoter in S. cerevisiae responds to nutritional signals including nitrogen, glucose and acetate. Mating-type control inhibits transcription of IME1 and IME4 via the non-coding RNAs IRT1 and RME2, respectively. Upon Rme1-dependent activation, the IRT1 transcript covers most of the IME1-promoter and blocks transcription factors from binding. RME2 is the antisense transcript of IME4 itself that is produced in haploid cells and blocks IME4 transcription. This mating-type controlled repression is relieved in diploid cells by the a1/a2 heterodimer that blocks IRT1 and RME2 transcription.

Ime1 activates transcription of IME2 that encodes a serine/threonine kinase (SMITH and MITCHELL 1989; YOSHIDA et al. 1990; KOMINAMI et al. 1993). Ime2 is required for activation of middle meiotic genes (MMG) that are essential for meiotic divisions and spore formation. Ime2 inactivates transcription of early meiotic genes by phosphorylating Ime1 that marks it for degradation by the proteasome. Ime2 also phosphorylates and inactivates the repressor of middle meiotic genes, Sum1, thus allowing transcription of NDT80 that encodes the main transcription factor required to activate middle meiotic genes (Figure 12).
**Ndt80** is the main regulator of MMG and binds to promoters harboring a consensus DNA sequence called the ‘middle sporulation element’ (MSE) (Winter 2012). Premature expression of MMG at the early stages of meiosis is prevented by the repressor Sum1 that recruits the histone deacetylase Hst1 to the **Ndt80**-promoter, thereby generating an inaccessible chromatin structure (Pak and Segall 2002; Neiman 2011). Inactivation of the Sum1 repressor by Ime2-mediated phosphorylation activates **Ndt80** expression by binding of the Ime1-Ume6 complex to the URS1 element (Figure 12) (Pak and Segall 2002).

![Diagram showing regulatory elements in meiosis in S. cerevisiae](image)

**Figure 12. The main regulatory elements involved in initiation of meiosis in S. cerevisiae.** Transcription of the early meiotic genes (EMG), including **IME2**, are initiated when Ime1 and Ume6 bind to the URS1 upstream controlling sequence of these genes. **IME2** encode a serine/threonine kinase that phosphorylates and inactivates Ime1 and Sum1, the repressor of the middle gene activator Ndt80. When Sum1 repression is relieved, Ime1 and Ume6 can activate transcription of **Ndt80**. Following activation, Ndt80 initiates transcription of middle meiotic genes (MMG) by binding to their middle sporulation element (MSE1). The middle meiotic genes are essential for meiotic division and spore formation (Sage et al. 1998; Pak and Segall 2002; Piekarska et al. 2010; van Werven et al. 2012).

In **paper II** we elucidate the roles of **IME1**, **IME2**, **IME4**, **Ndt80** and **Ume6** in regulation sporulation in *A. gossypii*.

**Role of glucose in regulating sporulation**

**S. cerevisiae**

The availability of nutrients such as nitrogen and glucose are regulating sporulation in *S. cerevisiae*. Glucose is a strong inhibitor of sporulation and as little as 0.2 % is sufficient for this block (Honigberg and Purnapatre 2003). The glucose signal is transmitted to the UCS2 element of the **IME1**-promoter via the PKA pathway. PKA is activated by the secondary messenger, cyclic adenosine monophosphate (cAMP) that is produced by adenylyl cyclase in response to different extracellular stimuli (D’Souza and Heitman 2001). In *S. cerevisiae*, PKA plays an essential role in nutrient sensing, while in various other fungi it regulates vegetative growth, sporulation, appressoria formation and mating (Lee et al. 2003). PKA is a holoenzyme composed of two regulatory subunits encoded by **BCY1** and two catalytic subunits that in *S. cerevisiae* are encoded by **TPK1**, **TPK2** and **TPK3**. Both subunits are highly conserved among fungi and other eukaryotes. In response to glucose, cAMP is produced and binds to Bcy1, thereby releasing the two active catalytic subunits (Toda et al. 1987). Active PKA then phosphorylates a number of downstream targets including the transcription factors Sok2, Msn2 and Msn4. Phosphorylated Sok2 represses **IME1** transcription while Msn2 and Msn4 are activators of
IME1 that are inhibited by PKA phosphorylation (Figure 13) (WARD et al. 1995; SAGEE et al. 1998; SHENHAR and KASSIR 2001).

A. gossypii

The components of the yeast PKA pathway are conserved in A. gossypii except for TPK3 that arose from the WGD in S. cerevisiae. The PKA pathway is involved in regulating both sporulation and riboflavin biosynthesis in A. gossypii since addition of a non-metabolisable cAMP analog blocks both the production of spores and riboflavin (STAHMMANN et al. 2001), yet the mechanisms behind this regulation remain unknown (Figure 13). The PKA pathway and its role in sporulation in A. gossypii via regulation of IME1 transcription is the main focus of paper I.

**S. cerevisiae**

Glucose

![Diagram]

**A. gossypii**

+ 5 mM cAMP

![Diagram]

Figure 13. Glucose regulation of sporulation via the PKA pathway in S. cerevisiae and A. gossypii. In response to glucose, cAMP is produced and binds to the regulatory subunits of PKA, encoded by BCY1 in S. cerevisiae. This activates the two catalytic domains, encoded by TPK1, TPK2 and TPK3 in yeast that phosphorylate the transcription factors Msn2, Msn4 and Sok2. Phosphorylated Sok2 blocks sporulation by repressing IME1 transcription while activation of IME1 via Msn2 and Msn4 is inhibited by PKA phosphorylation. When glucose is absent, the PKA pathway is inactive and Msn2 and Msn4 can enter the nucleus in their unphosphorylated form to activate expression of IME1. Msn2, Msn4 and Sok2 bind to the IME1 regulatory element IREu. In A. gossypii all the components of the PKA pathway are present except TPK3. Addition of exogenous cAMP blocks both sporulation and riboflavin production, however, the mechanism behind this remains unknown. From WASSERSTROM et al., 2014, manuscript.

**Meiotic recombination**

Production of spores through meiosis is a specialized cell division program where diploid cells reduce their number of chromosomes to produce haploid progeny. During meiosis, one round of DNA replication is followed by two rounds of cellular divisions. Prior to the first meiotic division, homologous chromosomes will pair and undergo recombination, a mechanism that increases the
genetic diversity in a population. Recombination also occurs in mitotically growing cells during repair of certain types of DNA damage, but it is much more frequent during meiosis. Many of the proteins used in mitotic repair are also required for meiotic recombination, suggesting that meiotic recombination evolved from mitotic recombination. However, key evolutionary events have led to several unique features in meiotic recombination that are well conserved among eukaryotes (Villeneuve and Hillers 2001). First, initiation of recombination is dependent on programmed double-strand breaks (DSBs) that are introduced by the conserved meiosis-specific endonuclease Spo11 (Keeney et al. 1997). Second, while crossover is avoided in mitotic cells to prevent chromosome rearrangement, it is promoted during meiosis. Third, mechanisms have evolved to ensure that meiotic recombination events occur between the two non-identical homologous chromosomes and not the two identical sister chromatids in order to increase genetic variability (Villeneuve and Hillers 2001; Kohl and Sekelsky 2013).

As previously mentioned, Spo11 is responsible for the deliberate induction of DSBs during meiotic recombination and orthologs are found in virtually all eukaryotes. Once DSBs have formed, the 5’ ended DNA is degraded to generate free 3’-ended single-stranded (ssDNA) tails. This DNA-processing event is carried out by a multisubunit DNA nuclease called the MRN-enzyme complex that consists of Mre11, Rad50 and Nbs1 (Xrs2 in S. cerevisiae). The ssDNA tails can invade the intact homologous DNA duplex and initiate DSB repair by priming DNA synthesis. This process is called strand invasion and requires two homologs of the bacterial RecA-family of DNA-exchange proteins, namely Dmc1 and Rad51. Dmc1 is a recombinase and together with Red1 and Hop1 it is also important to ensure that a majority of the meiotic recombination events occur between the two non-identical homologous chromosomes and not the two identical sister chromatids (Roeder 1997).

Repair of DSBs by homologous recombination can result in either crossover or noncrossover products, however, only crossovers segregate correctly during meiosis I. Therefore, a meiotic cell must ensure that recombination between homologs result in crossing over (Villeneuve and Hillers 2001). Meiotic crossover takes place in the so called synaptonemal complex, SC, a proteinaceous structure that form during meiosis and position homologs in close proximity. At the SC, crossovers are orchestrated by the widely conserved ZMM-family of proteins, an acronym for Zip1-4, Mrc3, Msh4/Msh5 and Spo16. Functional crossovers depend on the ZMM proteins and crossovers are reduced or absent in zmm mutants even though initiation of recombination is unaffected (Lynn et al. 2007). The Zip-proteins mediate protein-protein interactions while Msh4 and Msh5 form a heterodimer that stabilizes crossover-specific intermediates and thus actively promotes crossover.

**Meiotic recombination in A. gossypii?**

Sporulation in A. gossypii does not require mating and the fungus is able to sporulate abundantly in minimal media. However, one intriguing question remains unanswered: are spores derived from mitotic or meiotic divisions, i.e. does A. gossypii have a sexual cycle?

The fact that A. gossypii has lost all the genes encoding the ZMM-protein family except the Zip1 homolog suggests that meiotic crossover does not occur (Wendland et al. 2011). However, other meiosis specific genes like DMC1, SPO11, HOP1 and RED1 are present in the A. gossypii genome (Dietrich et al. 2004). In paper II we deleted SPO11 and DMC1 to determine if DSB formation by Spo11 and the subsequent repair by Dmc1 are required for sporulation in A. gossypii.
The cell wall integrity pathway

During my thesis I also worked with components of the cell wall integrity pathway (CWI) in *A. gossypii* as part of generating an *Ashbya* gene deletion library of 80 genes encoding components of signaling transduction pathways and various transcription factors.

*S. cerevisiae*

The CWI pathway found in yeast and many other fungi is required for cell wall remodeling during growth and in response to various stresses such as changes in extracellular osmolarity, growth temperatures and exposure to mating pheromone. The pathway is activated by a set of cell wall sensors including the integrin-like proteins Wsc1-3 (*VERNA et al. 1997*) and the O-glycosylated plasma membrane protein Mid2 (*KETELA et al. 1999*). These mechanosensors interact with the guanine exchange factor Rom2 to activate the small GTPase Rho1, which leads to activation of protein kinase C (Pkc1) (*PHILIP and LEVIN 2001*). The main role of activated Pkc1 is to trigger the MAP kinase module of the pathway. This module is activated through a phosphorylation cascade where Pkc1 phosphorylates the MAPKK Bck1 that activates the redundant MAPKKs Mkk1 and Mkk2, which, in turn, activate Mpk1/Slt2. The cascade terminates with expression of several transcription factors including Swi4, Swi6 and Rlm1 (Figure 14) (*LEVIN 2005*).

![Diagram of CWI signaling pathway](image)

**Figure 14. CWI signaling pathway.** Extracellular cell wall stress signals through the cell surface sensors Wsc1 and Mid2 to activate the Rho1 GTPase via its nucleotide exchange factor Rom2. Zeol1 that interacts with Mid2 in *S. cerevisiae* is absent in *Eremothecium* species. Rho1 activates protein kinase C (Pkc1), which subsequently activates the MAPK kinase module consisting of the MAPKK Bck1, the MAPKK Mkk1 and the MAPK Mpk1. The final targets of the CWI pathway are the transcription factors Swi4/Swi6 and Rlm1 (*LENGELER et al. 2013*).
A. gossypii and other fungi

The CWI pathway has not been investigated in A. gossypii, however, studies in other filamentous fungi have proven it to be important for pathogenesis. This includes the Mpk1 orthologs of several fungal species (Rispail et al. 2009). In C. albicans Mkc1 has important functions in cell wall integrity, growth at elevated temperatures and morphological transitions required for virulence (Diez-Orejas et al. 1997). In M. grisea, Mps1 is essential for conidiation, appressorial penetration, and plant infection (Xu et al. 1998). In F. graminearum, Mvg1 has essential function for enabling hyphal fusion and heterokaryon formation (Hou et al. 2002).

Final comments

In this thesis, a total number of 38 deletion strains have been generated and characterized. All these genes are presented in Table 2, together with a short description of their known function in S. cerevisiae. A schematic overview of the PCR-based gene targeting method is shown in Appendix I along with the verification PCRs of the deletion mutants. This has not been all my work and therefore I indicate in Appendix I which deletion strains were generated by me and by other members of the lab.
Table 2. Summary of the genes studied in this thesis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>S. cerevisiae gene</th>
<th>A. gossypii gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pheromone response cascade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gpa1</td>
<td>YHR005C</td>
<td>ADR153C</td>
<td>G-protein α-subunit</td>
</tr>
<tr>
<td>Ste4</td>
<td>YOR212W</td>
<td>ACR097W</td>
<td>G-protein β-subunit</td>
</tr>
<tr>
<td>Ste11</td>
<td>YLR362W</td>
<td>ABL011C</td>
<td>MAPKKK</td>
</tr>
<tr>
<td>Ste7</td>
<td>YDL159W</td>
<td>ACR196C</td>
<td>MAPKK</td>
</tr>
<tr>
<td>Fus3</td>
<td>YBL016W</td>
<td>AFR019W</td>
<td>MAPK</td>
</tr>
<tr>
<td>Dig1/2</td>
<td>YPL049C/YDR480W</td>
<td>AGL258C</td>
<td>Inhibitor of Ste12 and Tec1</td>
</tr>
<tr>
<td><strong>Karyogamy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kar1</td>
<td>YNL188W</td>
<td>ABL149W</td>
<td>Spindle pole body duplication</td>
</tr>
<tr>
<td>Kar2</td>
<td>YJL034W</td>
<td>ACR038W</td>
<td>ATPase – protein export to ER</td>
</tr>
<tr>
<td>Kar3</td>
<td>YPR141C</td>
<td>AGR253W</td>
<td>Kinesin motor protein, nuclear congression</td>
</tr>
<tr>
<td>Kar4</td>
<td>YCL055W</td>
<td>AFR736C</td>
<td>TF: required for KAR3 induction</td>
</tr>
<tr>
<td>Kar5</td>
<td>YMR065W</td>
<td>ADR143W</td>
<td>Nuclear fusion</td>
</tr>
<tr>
<td>Kar8</td>
<td>YJL073W</td>
<td>ADR124C</td>
<td>Nuclear fusion</td>
</tr>
<tr>
<td><strong>pH response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rim101</td>
<td>YHL027W</td>
<td>AFR190C</td>
<td>TF: pH response, reg sporulation</td>
</tr>
<tr>
<td><strong>PKA pathway</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tpk1/3</td>
<td>YJL164C/ YKL166C</td>
<td>AEL115C</td>
<td>PKA catalytic subunit</td>
</tr>
<tr>
<td>Tpk2</td>
<td>YPL203W</td>
<td>AFL090W</td>
<td>PKA catalytic subunit</td>
</tr>
<tr>
<td>Msn2/4</td>
<td>YMR037C/ YKL062W</td>
<td>ABR089C</td>
<td>TF: stress response</td>
</tr>
<tr>
<td>Sok2/Pdh1</td>
<td>YMR016C/ YKL043W</td>
<td>ABR055C</td>
<td>TF: sporulation, pseudohyphal differentiation</td>
</tr>
<tr>
<td><strong>Meiotic regulation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ime1</td>
<td>YJR094C</td>
<td>AGL308W</td>
<td>Activates early meiotic genes</td>
</tr>
<tr>
<td>Ime2</td>
<td>YJL106W</td>
<td>AFR076W</td>
<td>Protein serine/threonine kinase</td>
</tr>
<tr>
<td>Ime4</td>
<td>YGL192W</td>
<td>AFR173W</td>
<td>Methyltransferase required for spore induction</td>
</tr>
<tr>
<td>Ndt80</td>
<td>YHR124W</td>
<td>AGR347W</td>
<td>TF: activates middle meiotic genes</td>
</tr>
<tr>
<td>Ume6</td>
<td>YDR207C</td>
<td>AGL099C</td>
<td>TF: activates early genes with Ime1</td>
</tr>
<tr>
<td><strong>Sporulation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dmc1</td>
<td>YER179W</td>
<td>AGR101C</td>
<td>Repairs DBSs during meiosis</td>
</tr>
<tr>
<td>Spo1</td>
<td>YNL012W</td>
<td>AAL027W</td>
<td>Prospore protein</td>
</tr>
<tr>
<td>Spo11</td>
<td>YHL022C</td>
<td>ADR025W</td>
<td>Initiates DBSs during meiosis</td>
</tr>
<tr>
<td>Spo14</td>
<td>YKR031C</td>
<td>AFR071W</td>
<td>Phospholipase D required for meiosis</td>
</tr>
<tr>
<td><strong>Spore release</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eng1</td>
<td>YNR067C</td>
<td>AGL208C</td>
<td>Glucanase, mother-daughter separation</td>
</tr>
<tr>
<td>Eng2</td>
<td>YLR144C</td>
<td>AGL161C</td>
<td>Endoglucanase</td>
</tr>
<tr>
<td><strong>Cell integrity pathway</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wsc1</td>
<td>YOR008C</td>
<td>ADL020W</td>
<td>Sensor-transducer</td>
</tr>
<tr>
<td>Mid2</td>
<td>YLR332W</td>
<td>AEL302W</td>
<td>Sensor-transducer</td>
</tr>
<tr>
<td>Pkc1</td>
<td>YBL105C</td>
<td>ACR191C</td>
<td>Protein serine/threonine kinase</td>
</tr>
<tr>
<td>Tus1</td>
<td>YLR425W</td>
<td>AER110W</td>
<td>Guanine exchange factor (GEF) of Rho1</td>
</tr>
<tr>
<td>Bck1</td>
<td>YJL095W</td>
<td>AFR092W</td>
<td>MAPKKK</td>
</tr>
<tr>
<td>Mkk1/2</td>
<td>YOR231W YPL140C</td>
<td>ACR117W</td>
<td>MAPKK</td>
</tr>
<tr>
<td>Mpk1/Slr2</td>
<td>YHR030C/YKL161C</td>
<td>AER232C</td>
<td>MAPK</td>
</tr>
<tr>
<td>Swi6</td>
<td>YER111C</td>
<td>AGL297C</td>
<td>TF: reg. cyclins and DNA synthesis/repair</td>
</tr>
<tr>
<td>Rlm1/Smp1</td>
<td>YPL089C/ YBR182C</td>
<td>AGR198C</td>
<td>TF: maintenance of cell wall integrity</td>
</tr>
</tbody>
</table>

33
Summary of papers

Paper I

Analysis of the PKA pathway in Asbya gossypii reveals different modes of regulating sporulation that involves the positive elements Msn2 and Sok2

Glucose is a strong inhibitor of sporulation. Most of the glucose induced signaling in S. cerevisiae is transmitted via the conserved Ras/protein kinase A (PKA) pathway that regulate metabolism and growth. The PKA pathway controls morphogenesis and is therefore required for virulence in many pathogenic fungi. These morphological responses include the switch from yeast-like to filamentous growth, formation of appressoria and infection hyphae (KRONSTAD et al. 1998).

In paper I we study the role of the PKA pathway in regulating vegetative growth and sporulation in A. gossypii. We deleted the A. gossypii homologs to the two catalytic domains of PKA, Tpk1 and Tpk2, and the two transcription factors regulated by PKA that have a role in sporulation in S. cerevisiae, namely Msn2 and Sok2.

We show that the catalytic subunits have divergent functions in A. gossypii. Deletion of TPK1 results in decreased growth at 22, 30 and 37 °C, which suggests that Tpk1 is promoting vegetative growth in A. gossypii. On the contrary, deletion of TPK2 increases the radial growth at 37 °C and also partially at 30 °C. This might result from an increased availability of active Tpk1 when Tpk2 is absent. Moreover, Tpk2 is the main catalytic domain that transmits the glucose-signal to downstream targets since tpk2 spores are largely unable to germinate in response to glucose. Also, the germination efficiency does not improve in the tpk2 strain when exogenously cAMP is added that should activate PKA. Therefore, elevated levels of Tpk1 cannot compensate for the loss of Tpk2.

The PKA pathway in S. cerevisiae has an important role in transmitting the glucose signal to repress sporulation when glucose is present. During vegetative growth when glucose is available, PKA blocks IMEI expression via, (i) inhibition of the transcriptional activators Msn2 and Msn4, and, (ii) activation of the repressor, Sok2. Here we show that Msn2 and Sok2 have important functions in regulating sporulation also in A. gossypii. Deletion of MSN2 reduces sporulation and a sok2 mutant is completely non-sporulating. However, this regulation of sporulation by Msn2 and Sok2 does not seem to be via IMEI transcription.

Using a promoter deletion analysis we identified a minimal promoter including 491 bp of the upstream IMEI intergenic region to be sufficient to drive IMEI transcription. This is in sharp contrast to the >2.1 kb IMEI-promoter that is regulating transcription of IMEI in S. cerevisiae.

In a complementary approach we took the respective upstream DNA-regions of IMEI and fused them in-frame to the lacZ reporter gene. The resulting reporter genes were transformed into the A. gossypii wild type strain and the msn2 and sok2 mutants. β-galactosidase activity measurements show that the IMEI-promoter-lacZ fusions give similar activity in the msn2 and sok2 mutants as in the wild type strain. The promoter activity decrease in all three strains when 491 bp of the IMEI-promoter is used and drops even further using only 468 bp. This suggests that, in contrast to S. cerevisiae, Msn2 and Sok2 are actually not required for transcription of IMEI in A. gossypii. If they were we would have expected different expression levels of the promoter-lacZ constructs that contains binding sites for these transcription factors.
Finally, we report that the non-sporulating *ime1* mutant is overproducing riboflavin, which suggests that a block in the sporulation pathway can have a beneficial effect on riboflavin biosynthesis. A non-sporulating strain that halts the sporulation program at an early stage does not invest energy in the costly process of producing spores. However, this has not been seen in other non-sporulating mutants like *ime2, ime4, kar4* or *ndt80*, indicating that *Ime1* also has a role in regulating riboflavin biosynthesis.

**Paper II**

**Molecular determinants of sporulation in *Ashbya gossypii.***

Sporulation is a complex differentiation program that is induced in *S. cerevisiae* by starvation of nitrogen and glucose. These environmental signals activate a number of signaling pathways that lead to a transcriptional response resulting in the formation of spores. Sporulation in *A. gossypii* is also dependent on nutrient signals, however, the components involved in this regulation has not been determined.

In paper II we present an analysis of gene sets regulating sporulation in *A. gossypii*. We generated a set of 21 deletion mutants of conserved components known to be involved in sporulation in *S. cerevisiae*. To provide a comprehensive functional analysis of sporulation-specific components in *A. gossypii* we included genes involved in, (i) the pheromone/starvation MAP kinase cascades, (ii) nuclear fusion (karyogamy), (iii) initiation of sporulation, and, (iv) recombination.

Previously it has been reported that mating is not a prerequisite for sporulation in *A. gossypii* since a strain deleted for both pheromone receptors Ste2 and Ste3 is able to sporulate. However, heterologous expression of the AgSte2 α-factor receptor in a *S. cerevisiae ste2* strain results in growth arrest upon exposure to α-factor from *A. gossypii* (Wendland et al. 2011). This shows that the components of the *Ashbya* cascade are functional in transducing the pheromone signal. In this paper we show that deletion of the MAPKKK Ste11 and the MAPKK Ste7 of the pheromone/starvation MAPK pathways results in an oversporulating phenotype similarly to what is seen upon deletion of the transcription factors of the pathways, *STE12* and *TEC1*. Taken together, these results suggest that the pheromone/starvation cascades are used to regulate sporulation negatively in *A. gossypii* since over-sporulation occurs when components of the cascade are deleted.

Sporulation in *A. gossypii* is regulated at different levels by *IME1, IME2, IME4, KAR4* and *NDT80* since all these deletion strains are sporulation deficient. Using RNA sequencing we compared the transcript profiles of these non-sporulating strains with the wild type under sporulation-inducing conditions. We could identify a core set of 67 down-regulated genes in the *ime1, ime2, kar4* and *ndt80* mutants, most of which were up-regulated in the oversporulating *ste12* mutant. Out of these down-regulated genes, a core set of 28 genes could be identified that all harbored an Ndt80 binding site, RMCACAAAA, in their upstream intergenic regions.

Since mating is not needed for the production of spores in *A. gossypii*, we were interested in studying if karyogamy and recombination are required for sporulation. We analyzed the functions of the *A. gossypii* orthologs involved in karyogamy in *S. cerevisiae*, the so-called *KAR*-genes. Deletion of *KAR1* and *KAR2* in *A. gossypii* is lethal, which suggest that they have conserved vegetative functions similar to their *S. cerevisiae* homologs. Deletion of *KAR3* results in reduced growth at 37 °C but normal growth at 22
and 30 °C, indicating that Kar3 is not required for mitotic growth. However, the kar3 mutant is severely reduced in sporulation and the few spores that form are mostly unviable. Thus, our results suggest that Kar3 is in fact essential for karyogamy also in A. gossypii. The presence of karyogamy in A. gossypii is supported by the fact that a kar4 mutant is non-sporulating. In S. cerevisiae, Kar4 is a transcription factor that is induced in response to pheromone and is required for expression of KAR3.

The end result of mating and karyogamy in S. cerevisiae is a diploid cell that is able to go through the process of meiosis and sporulation to produce 4 haploid spores upon nutrient starvation. Meiotic recombination requires the formation and repair of double strand breaks (DSBs), which is controlled by Spo11 and Dmc1, respectively. In A. gossypii, DSB formation by Spo11 is not required for sporulation, yet, deletion of DMC1 that repairs DSBs in S. cerevisiae results in reduced sporulation. This suggests that A. gossypii can tolerate DSBs during sporulation, but Dmc1 is required the repair DSBs.

To summarize, with this study we provide a comprehensive overview of the genes involved in regulation of sporulation in A. gossypii. We also show that RNA sequencing is a powerful and reliable tool to study gene expression in this fungus.

**Paper III**

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

Fungal species live in a wide variety of habitats and are exposed to a number of environmental stresses including pH, nutrient limitations, changing osmolarity and temperature. A yeast cell living in the sugar rich environment on the skin of ripening fruit must be able to respond to sudden changes in osmolarity caused by for example a rainfall (Levin 2005). This requires a rigid cell wall that needs to be remodeled and repaired in response to external challenges that cause cell wall stress. Theses alterations of the fungal cell wall are orchestrated by the cell wall integrity pathway, abbreviated CWI. The CWI pathway is comprised of a set of G-protein coupled cell wall sensors that activate a MAP kinase cascade. The cascade terminates at several transcription factors that facilitate biosynthesis of cell wall components and actin organization (Fuchs and Mylonakis 2009).

In paper III we present a comprehensive functional analysis of the A. gossypii CWI pathway. A total set of ten deletion mutants were generated of conserved components including the cell surface sensors AgWSC1 and AgMID2, the putative Rho1-guanine nucleotide exchange factor AgTUS1, the protein kinase C, AgPKC1, the MAPK-kinase cascade including AgBCK1, AgMKK1 and AgMPK1 and the transcription factors regulated by the pathway AgRLM1, AgSWI4 and AgSWI6.

The results show a pronounced cell lysis phenotype in many of the mutant strains, including wsc1, pck1 and the MAP kinase mutants. Moreover, deletion of WSC1 resulted in reduced growth and deletion of PKC1 generated mutants that showed high degree of tip lysis. Also in the mpk1 mutant strain tip lysis occurred but at a lower rate that did not affect radial growth.

One idea behind this study was to use the colony lysis phenotype of the CWI mutants to facilitate isolation of riboflavin from A. gossypii mycelium. As previously described, A. gossypii overproduces riboflavin and the fungus is used for industrial production since riboflavin has commercial value as an additive and colorant in the food industry. Riboflavin is produced at the end of the growth phase and is
stored to a large extent in the vacuoles. Harvesting of riboflavin might therefore be facilitated in CWI mutants that promote cell lysis at this stage of development.

To test the cytoplasmic leakage of the CWI mutants, alkaline phosphatase and β-galactosidase release assays were used. The results show that the MAP kinase mutants bck1, mkk1 and mpk1 have strong cytoplasmic leakage while intermediate levels of leakage is seen in the usc1 and mid2 mutants. Interestingly, deletion of WSC1 resulted in a strong increase of riboflavin in the growth medium suggesting that cell lysis occurs at a favorable time point regarding riboflavin production. On the contrary, the MAP kinase mutants did not display this feature indicating that colony lysis occurs too early to allow riboflavin overproduction.

To conclude, our study in paper III provides new targets that allow for both the improvement of riboflavin biosynthesis and provide means of isolating riboflavin on an industrial scale.

**Paper IV**

**Role of pH in regulating sporulation in Ashbya gossypii**

All microorganisms are exposed to large fluctuations in pH in their surrounding environment. Therefore it is critical for their survival to be able to sense and respond to these changes in pH. Extracellular pH has been shown to be a key signal for differentiation pathways important for virulence including the morphological switch from yeast-like to filamentous growth and sporulation. The response to pH in many fungal species is mediated by a conserved signaling pathway that results in activation of the transcription factor Rim101/PacC. Gene regulation by ambient pH has been most widely studied in *A. nidulans* where PacC activates alkaline-expressed genes and represses acid-controlled genes in response to alkaline pH. In *S. cerevisiae*, the PacC homolog Rim101 is required for adaptation to extracellular pH and to regulate transcription of *IME1*. In both *S. cerevisiae* and *A. nidulans*, Rim101 and PacC are important for growth at alkaline conditions since they regulate the developmental processes of sporulation and conidiation in these fungi, respectively.

In paper IV we investigate the role of the *A. gossypii* Rim101 homolog in adaptation to alkaline pH. Our results show that deletion of *AgRIM101* has no effect on vegetative growth or sporulation under normal growth conditions. However, Rim101 is important for adaptation to alkaline pH also in *A. gossypii*. The radial growth is decreased in the *rim101* mutant at alkaline pH when compared to the wild type, which is particularly pronounced at pH 8.0-8.5. The *rim101* colonies are also less dense at alkaline pH and a lysis zone form. As described above, alkaline pH is not required for sporulation in *A. gossypii* as it is in *S. cerevisiae*, yet, Rim101 appears to be needed for wild type levels of sporulation at alkaline pH. We isolated spores from colonies grown at different pH and determined the spore forming units in the *rim101* mutant and the wild type. The *rim101* mutant sporulates like wild type at pH 6.5, but the spore production is decreased at pH 7.5-8.5. This could also be visualized by looking at the size of the sporulation zone on the plates.

In this study we begin to investigate the role of Rim101 in regulating growth and sporulation in response to alkaline pH in *A. gossypii*. We are able to show that Rim101 is not required for spore production under normal sporulating-inducing conditions. Yet, the functions of Rim101 regarding
adaptation to alkaline pH seems to be conserved in *A. gossypii* since both growth and sporulation are decreased at pH above 7.5 in the *rim101* mutant.

**Paper V (not included in this thesis)**

**Fungal model systems and the elucidation of pathogenicity determinants.**

Fungal species are able to cause devastating disease both on plants and in animals, and therefore have a large negative impact on both agriculture and human health. Every year, fungal infections threaten both food security and animal health with large crop losses and high mortality rates. The need for new antifungal drugs is therefore critical, however, this requires great molecular knowledge of the components controlling fungal virulence. In this review we present the current understanding of seven fungal model organisms used for studying fungal pathogenesis. We include the two major human fungal pathogens, *A. fumigatus* and *C. albicans*, the plant pathogenic species, *F. oxysporum*, *M. oryzae*, and *Z. tritici* as well as the two model organisms of filamentous and dimorphic fungi, *A. gossypii* and *U. maydis*. The purpose of the review is to present key insights into mechanisms of pathogenesis employed by these fungal species and also to provide a comparative overview of key insights learned from genomic analysis. By comparing some of the best-studied fungal pathogens, this review highlights both specific differences and similarities between the species, thereby generating better understanding of current trends and future challenges considering fungal pathogenicity.
Conclusions and future perspectives

Sporulation is a key developmental process that enables fungi and other organisms to survive harsh environmental conditions. While asexual sporulation results in the formation of large numbers of spores that are genetically identical, sexually derived spores introduce the possibility of genetic variation in a population.

At the onset of this study it was not known whether *A. gossypii* has a sexual cycle or not. Many mating and meiosis-specific genes known from yeasts are present in the *A. gossypii* genome. This includes genes involved in, (i) the pheromone response pathway, (ii) karyogamy (KAR-genes), (iii) regulating initiation of sporulation (IME-genes) and, (iv) meiotic recombination (SPO11, DMCI). Yet, some important meiotic determinants are absent, including the synaptonemal complex (SC) components ZIP2-4 and MSH4/MSH5 that are required for normal levels of crossover during meiosis in *S. cerevisiae*. In this thesis I have investigated the role of some of these meiotic-specific components in *A. gossypii* and I believe this has provided further genetic insight into the sporulation process in this fungus. The main findings of my work in *A. gossypii* are discussed in the following sections.

The pheromone response pathway is regulating sporulation negatively in *A. gossypii*

In this thesis I confirm a paradigm shift in the role of the pheromone response pathway in *A. gossypii*. In this fungus the pathway is regulating sporulation negatively since deletion of several components including STE11, STE7, STE12 and TEC1 results in oversporulating phenotypes. This is in contrast to yeast where disruption of components of the pathway would result in sterile mutants. Interestingly, however, the function of the cascade is conserved in *A. gossypii* since a constitutively active allele of the MAPKK Ste7, so called Ste7\textsuperscript{act}, results in a mating response in *S. cerevisiae* (paper I). Furthermore, our study in paper II show that deletion of the G-protein subunits GPA1 and STE4 has no effect on growth or sporulation in *A. gossypii*. This is in sharp contrast to *S. cerevisiae* where GPA1 is an essential haploid specific gene for cell-growth (MIYAJIMA et al. 1987). Thereby we conclude that the function of the pheromone response pathway have been rewired in *A. gossypii* since it is not essential for growth or sporulation. We speculate that the specialized life style in the gut of the insect might have promoted this rewiring to allow sporulation even in the absence of a mating partner. Furthermore, the oversporulating phenotypes of the deletion mutants indicate that the function of the pathway has been adapted to promote sporulation as a default rather than using mechanisms to prevent sporulation.

Karyogamy functions of Kar3 and Kar4 seems to be conserved

A prerequisite for sexual reproduction is karyogamy in order to generate diploid nuclei able undergo meiotic recombination. In paper II we deleted all the conserved KAR-genes in *A. gossypii*, which resulted in two mutants with sporulation defects, namely kar3 and kar4. In *S. cerevisiae*, Kar3 is a kinesin-like motor protein that is essential for nuclear congression during meiosis. Transcription of ScKar3 is in fact activated by the pheromone-induced ScKar4. In the scope of this thesis I was not able to determine whether the sporulation phenotype is due to defective karyogamy or if AgKar3 and AgKar4 have other roles in sporulation. Therefore a future key experiment will be to determine if, when and how a nucleus becomes diploid in *A. gossypii.*

39
Karyogamy can be studied in forthcoming experiments by generating heterokaryotic mycelia with nuclei that harbor either one of two fluorescent proteins. Nuclear fusion of two such nuclei containing different fluorescent proteins will result in a diploid nucleus that harbors both (Figure 15). If nuclear fusion occurs it will be interesting to compare the nuclei migration and positioning in the wild type strain with the kar3 and kar4 mutants. This type of study requires a system for live microscopy under sporulating conditions to be developed. One slightly complicated aspect of the experiment is that the heterokaryotic mycelia cannot be generated by mating since this has not been observed in A. gossypii. Instead, the two markers should be transformed simultaneously and designed to integrate at the same genetic locus in order to allow only one fluorescent marker per haploid nuclei. This should generate hyphae containing a mix of nuclei that has either one of the two markers integrated or no marker at all.

![Figure 15. Experiment to study karyogamy in A. gossypii.](image)

**Figure 15. Experiment to study karyogamy in A. gossypii.** (A) Heterokaryotic mycelia containing nuclei with either a red or a green fluorescent marker or no marker at all. (B) Karyogamy should give rise to nuclei that contain both fluorescent markers. This scenario is shown as an orange colored nucleus in the schematic description (indicated by a black arrow).

**Main regulators of sporulation are IME1, IME2, IME4, KAR4, NDT80 and UME6**

Initiation of sporulation in *S. cerevisiae* is regulated by a number of meiosis-specific genes activated in response to nutrient starvation. In paper II we generated deletion strains in *A. gossypii* of these specific regulators of sporulation, namely *IME1, IME2, IME4, KAR4, NDT80* and *UME6*. The resulting deletion mutants were all non-sporulating except for *ume6*. We could show that the hierarchical organization of these components is most likely conserved in *A. gossypii*. The sporulation phenotype of AgIme4 and AgKar4 indicate that they act upstream of AgIme1 and AgIme2, just like in *S. cerevisiae*. In the *ime1* and *ime2* mutants more or less all the mycelial compartments swell and form sporangia in sporulating media while the *ime4* and *kar4* mutants still contain vegetative mycelia that have not entered the sporulation program.

In paper II we also identified a set of 67 down-regulated genes in the *ime1, ime2, kar4* and *ndt80* mutants by using RNAseq under sporulation-inducing conditions. Interestingly, the same set of genes was up-regulated in the oversporulating ste12 mutant. Out of these down-regulated genes, a core set of 28 genes could be identified that all harbored a Ndt80 binding site, RMCACAAAA, in their upstream intergenic regions.
Differential regulation of AgIME1 vs ScIME1

The AgIME1-promoter deletion analysis in paper I allowed us to identify a minimal promoter of 491 bp to be required for wild type expression of AgIME1. This is intriguing since IME1 in S. cerevisiae is regulated by a >2.1 kb promoter. Interestingly, we provide evidence that expression of IME1 is not regulated by Msn2 and Sok2 even though the AgIME1-promoter contains putative binding sites for Msn2 at -512 and for Sok2 at -490. A future key experiment will be to identify the transcription factors that actually interact with the IME1-promoter. One candidate to investigate would be Adr1 since there is a putative binding site for this protein at -480. Adr1 has recently been shown to have a role in IME1 regulation in S. cerevisiae (Young et al. 2003). This type of study would require Chromatin Immunoprecipitation Sequencing, ChIPseq, which allows identification of DNA regions a specific transcription factor binds to. The drawback of this technique is that only known transcription factors can be studied. In our case it would actually be more interesting to identify all transcription factors that bind to the AgIME1-promoter.

Msn2 and Sok2 are important regulators of sporulation but not via IME1 transcription

The IME1-promoter in A. gossypii does not appear to be regulated by Msn2 and Sok2 as shown in paper I. However, since both Msn2 and Sok2 have important roles in sporulation in A. gossypii they must control transcription of other genes. One might speculate that Sok2 actually has a more prominent role than Ime1 in regulating sporulation in A. gossypii and might in fact be the master transcriptional regulator of sporulation. This would be in sharp contrast to S. cerevisiae where Ime1 is the central component regulating sporulation. Interestingly, despite lacking IME1 a complete sexual cycle has been reported in the hemiascomycete Candida lusitaniae. In this fungus the programs regulating mating and meiosis have fused and the pheromone response pathway is induced throughout the sexual cycle. In the absence of IME1, the functions of IME2 and STE12 have been rewired to include both mating and meiosis in C. lusitaniae. In contrast to S. cerevisiae that is predominantly diploid in nature, C. lusitaniae cells only exhibit a transient diploid state and preferentially exist in the haploid state. The haploid life style is thought to be promoted by the coupling of mating and meiosis (Sherwood et al. 2014). Similarly, A. gossypii exists predominantly in the haploid state and to this date a diploid life style has not been identified. The haploid life style in A. gossypii might be promoted in a different manner by enabling sexual reproduction without the need for mating. Thus the diploid state might occur only shortly during sporulation, soon after which the haploid state is regained. Our results show that sporulation in A. gossypii is regulated mainly via nutritional signals like glucose and nitrogen, which could have resulted in a more prominent role for Sok2 in transcriptional regulation.

Further experiments have to be carried out to investigate the promoter regions regulated by Msn2 and Sok2. The method of choice would be ChIPseq since it identifies the DNA regions a particular transcription factor binds to. RNAseq of the deletion strains msn2 and sok2 would also be useful to identify the specific genes they transcriptionally regulate. The drawback with RNAseq is that this technique also identifies indirect target genes whose expression is altered as a consequence of the gene deletion and not because direct regulation by the transcription factor.
Spo11 and Dmc1 are not required for sporulation

Meiotic recombination of homologous chromosomes is facilitated by a series of programmed DSBs carried out by Spo11 in *S. cerevisiae*. After recombination, these DSBs have to be repaired, which in yeast requires Dmc1. In paper II we show that neither of these proteins is necessary for sporulation in *A. gossypii*. This could either mean that DSB formation and repair is carried out independently of Spo11 and Dmc1 or that meiotic recombination does not occur.

Future experiments to investigate the presence of meiotic crossover in *A. gossypii* could again include a heterokaryotic strain that harbor nuclei with either one of two integrated markers. However, in this experiment the integration sites of the two markers should be at different loci but on the same chromosome. Following karyogamy, meiotic recombination should generate a chromosome that contains both markers. This can be proven by plating the resulting haploid spores on double selective media to only allow growth of haploid homokaryons that contain both antibiotic markers. However, the real difficult part with this experiment will be to ensure that the transformed mycelia only has one integrated marker per nucleus before the start of the crossover experiment.

Final conclusions

Our studies in *A. gossypii* together with previous findings suggest that the sporulation program has been rewired, and mechanisms have developed to promote sporulation rather than to inhibit it. The specialized life style in the gut of the insect might have put strong selection pressure on the ability to sporulation even in this environment. We refer to this rewiring of the sporulation program in *A. gossypii* as a ‘paradigm shift’ that is unique among the *Saccharomyces* and involves a number of features. (1) The pheromone/starvation pathway regulates sporulation negatively, thus minimizing the need for pheromone to produce spore. (2) *RME1* is absent in the *A. gossypii* genome and therefore repression by the α1/α2 heterodimer is not required for sporulation. (3) Sok2 functions as an activator and not a repressor of sporulation.

The multinucleate compartments of *A. gossypii* might have enabled the fungus to go through karyogamy and meiosis without the need for a mating partner, however, a sexual cycle has not been found yet. We speculate that sporulation in *A. gossypii* could result from unisexual reproduction that has recently been discovered in both homothallic ascomycetes and basidiomycetes. Unisexual reproduction gives the advantage of genetic exchange and recombination in the absence of the opposite mating type. In the fungal pathogen *Cryptococcus neoformans*, unisexual reproduction provides a dispersal advantage through hyphal growth and sporulation, which might enable the fungus to easier access nutrients and find a potential mating partner (Phadke et al. 2013).

The fact that Spo11 and Dmc1 are not required for sporulation in *A. gossypii* make room for speculation that meiotic recombination is carried out by other components. Meiotic recombination in *S. cerevisiae* and most other eukaryotes involve the formation of a synaptonemal complex (SC) that facilitates pairing of homologous chromosomes. This requires the ZMM (Zip1-4, Msh4/Msh5 and Mer3) family of proteins. However, some fungal species are able to go through meiosis without formation of SC. This includes the sexually reproducing fungi *Schizosaccharomyces pombe* and *C. lusitaniae*. In *C. lusitaniae*, meiotic recombination occurs with the same frequencies as other sexual fungi even though many of the key meiotic components required for efficient crossover are absent including Dmc1, the SC proteins Zip1-4 as well as Msh4/Msh5 and Mer3. However, meiotic
recombination in both *S. pombe* and *C. lusitaniae* is dependent on Spo11 (Sherwood and Bennett 2009). This shows that even though *A. gossypii* lack most of the ZMM components, meiotic recombination could still occur. Other work in the basidiomycete *Coprinus cinereus* show that a mutation in Nbs1, a core component of the Mre11-Rad50-Nbs1 complex, results in meiotic crossover and chromosomal segregation that is independent of Spo11 (Crown et al. 2013). Thus, other components than Spo11 can initiate DSBs.

Meiosis can also be carried out through other ways than conventional meiosis. The parasexual cycle of *C. albicans* involve efficient mating that produces tetraploid a/α cells. These cells return to the diploid state through random chromosome loss that does not involve recombination but requires both Spo11 and Dmc1. It has been speculated that an advantage of the parasexual program might be that the reduced number of spores could be beneficial in the host since spores are often highly antigenic (Sherwood and Bennett 2009).

Based on all these findings in other fungal model organisms, it is possible that meiotic recombination occurs also in *A. gossypii* even though many of the key components are missing or have other functions. All other *Saccharomycetaceae* sporulate by producing meiotic offspring and therefore it is my belief that also *A. gossypii* does so, we just need to unravel the mechanisms behind it.
References


KETELA, T., R. GREEN and H. BUSSEY, 1999 Saccharomyces cerevisiae mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. J Bacteriol 181: 3330-3340.


PHILIP, B., and D. E. LEVIN, 2001 Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. Mol Cell Biol 21: 271-280.


WENDLAND, J., and A. WALTHER, 2011 Genome evolution in the eremothecium clade of the Saccharomyces complex revealed by comparative genomics. G3 (Bethesda) 1: 539-548.


Appendix I

PCR based disruption cassettes were amplified from pFA-GEN3 (499/C634) using S1- and S2 primers that contained 50 bp homologous flanks to ‘your favorite gene’. The PCR product was precipitated and transformed directly into *A. gossypii* using electroporation. Diagnostic PCR was used for verifying correct integration of each disruption cassette at the desired locus. Gene specific primers annealing upstream (G1) and downstream (G4) of the marker integration site were used together with the two *GEN3* primers, G2 (1214 or 1112) and G3 (1215 or 1113). The absence of the target gene in homokaryotic deletion strains were verified using the internal I1/I2 primers.

![Diagram](image)

Figure 1. PCR-based gene targeting in *A. gossypii*.

After transformation, correct integration in the heterokaryotic strains was verified by PCR. The heterokaryons were then sporulated and by micromanipulating the haploid spores onto selective media, homokaryotic mutants were selected.
Mutants generated by Lisa Wasserstrom.
Mutants generated by Lisa Wasserstrom.
Mutants generated by Lisa Wasserstrom.
Mutants generated by Lisa Wasserstrom.

Δtpk2

G1-G2  I1-I2
1  2  3  4  5
1 AWL182  C914  leu2 tpk2::GEN3 het
2 AWL183  C915  leu2 tpk2::GEN3 het
3 AWL184  C916  leu2 tpk2::GEN3 hom
4 AWL185  C917  leu2 tpk2::GEN3 hom
5 Δtpk2

G3-G4 could not be verified

Δume6

G1-G2  G3-G4  I1-I2
1  2  3  4  5
1 AWL29  C436  leu2 ume6::GEN3 het
2 AWL30  C437  leu2 ume6::GEN3 het
3 AWL67  C515  leu2 ume6::GEN3 hom
4 AWL68  C516  leu2 ume6::GEN3 hom
5 Δume2

Mutants generated by Klaus B. Lengeler

Δeng1

G1-G2  G3-G4  I1-I2
1  2  3  4  5
1 ALK36-1  C665  leu2 eng1::GEN3 het
2 ALK36-2  C666  leu2 eng1::GEN3 het
3 ALK36-10  C667  leu2 eng1::GEN3 hom
4 ALK36-20  C668  leu2 eng1::GEN3 hom
5 Δeng1

Δeng2

G1-G2  G3-G4  I1-I2
1  2  3  4  5
1 ALK37-1  C669  leu2 eng2::GEN3 het
2 ALK37-2  C670  leu2 eng2::GEN3 het
3 ALK37-20  C671  leu2 eng2::GEN3 hom
4 ALK37-20  C672  leu2 eng2::GEN3 hom
5 Δeng2

Δime1

G1-G2  G3-G4  I1-I2
1  2  3  4  5
1 AL444-1  C743  leu2 ime1::GEN3 het
2 AL444-2  C744  leu2 ime1::GEN3 het
3 AL444-10  C745  leu2 ime1::dime3 hom
4 AL444-20  C746  leu2 ime1::dime3 hom
5 Δime1
Mutants generated by Klaus B. Lengeler

\[ \Delta m2 \]

\[ \Delta mkk1 \]

\[ \Delta msn2 \]

\[ \Delta ndt80 \]

\[ \Delta pck1 \]

\[ \Delta lin1/smp1 \]
Mutants generated by Klaus B. Lengeler

Δslt2/mpk1

Δspo1

Δste7

Δste11

Δswt4

Δtus1
Mutants generated by Klaus B. Lengeler

\[ \Delta \text{wsc1} \]

\[
\begin{array}{ccc}
\text{G1-G2} & \text{G3-G4} & \text{I1-I2} \\
1 & 1 & 1 \\
2 & 2 & 2 \\
3 & 3 & 3 \\
4 & 4 & 4 \\
5 & 5 & 5 \\
\end{array}
\]

400 bp 330 bp 655 bp

\[ \Delta \text{dig1} \]

\[
\begin{array}{ccc}
\text{G1-G2} & \text{G3-G4} \\
1 & 1 \\
2 & 2 \\
3 & 3 \\
4 & 4 \\
5 & 5 \\
\end{array}
\]

381 bp 380 bp

1 ALX5-1 CB32 leu2 dig1::GEN3het
2 ALX5-2 CB33 leu2 dig1::GEN3het
3 ALX5-10 CB34 leu2 dig1::GEN3hom
4 ALX5-20 CB35 leu2 dig1::GEN3hom
5 leu2

11/12 primers did not work, therefore the dig1 mutant was verified by complementation with the full-length DIG1 gene.