Final Report

Many species of kingdom Fungi alternate between two or more types of growth morphology. The di- and polymorphic human and animal pathogenic fungi usually use filamentous forms (true hyphae or pseudohyphae) for invading solid substrates (e.g. tissues) and single-cell forms (yeasts or yeast-like cells) for propagating in liquid environment. The most intensively studied polymorphic fungi are the human pathogenic *Candida* yeasts. Morphological polymorphism is also quite widespread among plant pathogens (e.g. *Ustilago*) and among saprophytic species (e.g. many nonpathogenic *Candida* species, *Schizosaccharomyces*, *Jaminaea*, *Mucor*).

The yeast-to-filamentous and the filamentous-to-yeast transitions are gradual events that involve drastic reprogramming of many cellular processes: growth polarity determination, intracellular trafficking, cell wall synthesis, vacuolation, cell size determination, septum cleavage (cell separation), etc. Yeast cells grow isotropically (budding yeasts) or bipolarly (fission yeasts), whereas the hyphal cells grow unipolarly and are usually longer (larger) than the yeast cells. Hyphal cells are prone to develop larger vacuoles usually located at the non-growing cell part. The septum produced at the end of the cell cycle to separate the sister cells is cleaved in the yeast phase but remains intact in the hyphal phase.

The non-pathogenic fission yeast *Schizosaccharomyces japonicus* alternates between filamentous and yeast growth phases, and its morphological transitions are regulated by external signals much as in many pathogenic fungi. Due to this similarity, *S. japonicus* can be used as a non-pathogenic model (safer for laboratory work) for studying the processes underlying the morphological versatility which is an important pathogenesis factor in many fungal species. In this project, genes involved in the separation of the daughter cells upon septation (cytokinesis) were investigated. This process is active in yeast cells but becomes inactivated at the transition from the yeast phase to the filamentous (hyphal, pseudohyphal) phase.

The project had three work tasks subdivided in smaller tasks scheduled for three years. All objectives were achieved, but we had to request a no-cost extension (8 months) of the project period because of the delayed updates of the genome annotations of certain yeasts. We also requested permissions for involving more students in the work and for purchasing a computer to improve the hardware facilities used in the bioinformatics part of the project.

1. Identification and characterization of putative orthologues of *S. pombe* cell separation genes in other fungal species by comparative in-silico analysis

1.1.Identification of putative orthologues of *S. pombe* cell-separation genes in other fungi

1.1.1. Fungal species with sequenced genomes: reciprocal best-hit search with *S. pombe* translated sequences in the genomes of the other species and vice-versa

1.1.2. In species with incompletely sequenced genomes: Identification of putative orthologues with *S. pombe* translated sequences in NCBI databases and testing them for best similarity in the *Schizosaccharomyces* genome databases.

Results: We searched for orthologues of the *S. pombe* (a non-dimorphic sibling species of *S. japonicus*) cell separation genes *ace2, agn1, eng1* and *sep1* in the 126 completely sequenced fungal genomes available at that time in the NCBI genome sequence database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi) or in the Broad database (http://www.broadinstitute.org//scientific-community/data) with the reciprocal best-hit search method. These databases contained the genome sequences of many Basidiomcota, Pezyzomycotina and Saccharomycotina species but only four Taphrinomycotina species. As

the latter subphylum harbours the dimorphic *S. japonicus* and its sibling species (*S. pombe, S. octosporus* and *S. cryophilus*), we needed orthologues from more Taphrinomycotina species for the phylogenetic analysis. Therefore we also searched the incomplete genome sequences of *Saitoella complicata* and *Pneumocystis carinii* available at http://genome.jgi.doe.gov/pages/blast.jsf?db=Saico1 and http://pgp.cchmc.org/, respectively. (See references 1, 4, 5 and 6).

1.2. Selection of non-*S. pombe* genes (e.g. genes of *S. japonicus* and *Candida albicans*) for cloning and molecular examination

Results : The *S. japonicus* and *C. albicans* genes encoding the most similar counterparts of ace2Sp, sep1Sp, agn1Sp and eng1Sp (Sp stands for *S. pombe*) proteins were selected for cloning and molecular examination. Remarkably, *C. albicans* had no orthologue of agn1Sp. As this species belongs to Saccharomycotina, we checked all genome sequences of the Saccharomycotina subphylum for agn1Sp orthologues. None of them had agn1 genes. We hypothesize, that the Saccharomycotina branch lost this gene (coding for alpha-glucanase) after its separation from the rest of Ascomycota, simultaneously with the loss of alpha-glucan synthesis in the cell wall. (See references 1, 5 and 6)

1.3. Comparative analysis to identify variable and conserved regions (domains) of the translated gene products.

Results: We identified conserved domains in the Ace2p (Zn-finger DNA-binding domain), Sep1p (forkhead-type DNA-binding domain), Agn1p (GH71: Glycoside hydrolase family 71 domain) and Eng1p (GH81: Glycoside hydrolase family 81 domain) proteins in all *Schizosaccharomyces* species. Agn1p and Eng1p also had N-terminal secretion signals and *Schizosaccharomyces*-specific C-terminal conserved blocks. The Eng1p proteins also possessed carbohydrate binding domains consisting of three short repeats (see references 1, 5 and 6).

1.4. Phylogenetic analysis of entire proteins and conserved domains to map the evolutionary relationships of cell separation genes in dimorphic, polymorphic and morphologically stable species.

Results: The fungal orthologues of the *S. pombe* cell separation genes/proteins were found highly variable in size, mainly due to the diversity of the terminal regions that flanked the conserved domains. Therefore we performed the phylogenetic analysis only with the conserved domains. The Zn-finger domains of the Ace2 and the forkhead domains of the Sep1 transcription factors reflected the phylogenetic relationships among the examined species. Agn1 was an exception because the *Schizosaccharomyces* Agn1 proteins were somewhat closer to their Basidiomycota counterparts than to their Pezyzomycotina orthologues. (See references 1, 5 and 6). In a separate line of research, we noticed a correlation in Kingdom Fungi between the diversification of the of Zn-finger domain transcription factors and the ability to split the septum upon the completion of cytokinesis. The septum remains intact in the multicellular hyphae of filamentous fungi and in the filamentous stage of the dimorphic species (e.g. *C. albicans, S. japonicus*) and will be split in the yeasts. We hypothesized that this diversification is essential for the adoption of the yeast mode of propagation. (See reference 4).

2. Exploring the functions of the *S. japonicus* genes identified by the in-silico analysis

2.1. Construction of geneticin-resistance deletion casettes flanked with short sequences corresponding to regions of the *S. japonicus* target genes

Results : For the inactivation of the *ace2Sj*, *sep1Sj*, *agn1Sj* and *eng1Sj* (Sj stands for *S. japonicus*) genes, we first localized them in the *S. japonicus* genome, then we designed primers for the amplification of the sequences that flanked their coding regions in the chromosomes . Using the amplified sequences and a geneticin-resistance cassette (developed for gene replacement in *S. pombe*), we amplified constructs consisting of the upstream and

downstream flanking sequences of the genes and the resistance cassette inserted between them. These constructs can replace by homologous recombination the coding regions of the target genes in the *S. japonicus* chromosomes (see reference 1).

2.2. Vector construction for regulated heterologous expression of genes in *S. japonicus* (episomal vectors and expression vectors are not available yet for this species)

Results : In a previous project we cloned a chromosomal segment from *S. japonicus* which showed a weak *ars* activity when inserted into plasmids and transformed into *S. japonicus* cells . Recently, a Japanese laboratory constructed another plasmid with a different *ars*-like chromosomal fragment. Unfortunately, neither our vector nor their plasmid could be maintained extrachromosomally in the *S. japonicus* transformants. Therefore we did not use episomal vectors for the heterologous expression of foreign genes in S. japonicus. Instead we integrated expression constructs containing the coding regions of the genes fused with controllable *nmt* promoters into the chromosomes by the gene-replacement method (see references 1, 5 and 6). For this, we developed gene-replacement expression cassettes based on geneticin-resistance.

2.3. Deletion of *S. japonicus* genes with cassettes constructed in Task 2.1.

Results: The genes coding for the proteins analysed in work task 1.3 were reamplified from the *S. japonicus* genome. The fragments obtained were disrupted by inserting geneticin-resistance cassettes into the coding sequences. The interruption constructs were transformed into the wild-type *S. japonicus* cells. After transformation, geneticin-resistant colonies (putative integrative transformants) were selected and checked for the chromosomal locations of the integrated casettes. Colonies in which the wild-type genes were replaced with the interruption constructs were isolated for further examinations. (See references 1, 5 and 6).

2.4. Examination of the deletion mutants of *S. japonicus* (constructed in Task 2.3) for cell separation in the yeast phase and for morphological transitions

Results: All deletion mutants were tested for deficiencies in the process of post-cytokinetic cell separation (septum cleavage) by fluorescence microscopic and transmission electron microscopic examination. In the yeast phase of the dimorphic *S. japonicus*, the ace2- and sep1- mutants were almost completely defective in septum cleavage; the transformants formed branching cell chains. The cell chains stained with a primary-septum specific brightener had bright septa in the fluorescence microscope, demonstrating that the mutant cells could not degrade their primary septa. Intact primary septa were also seen in the electron-microscopic images. The dissolution of the material of this layer of the septum is essential for cell separation. The mutants defective in *agn1* or in *eng1* only showed mild separation defects. Since *S. japonicus* alternates between mycelial and yeast growth phases, we also examined the effect of the inactivation of the genes on the yeast-to-hypha and the hypha-to-yeast transitions as well as on the growth of hyphae. Defects were detected only at the hypha-to-yeast transition. (See references 1, 5, 6 and 7). These transitions were examined in agar films because true hyphae are not produced in liquid substrates (see reference 2).

2.5. Integration of *S. pombe* cell separation genes and certain (*Candida* and *Saccharomyces*) genes cloned in Task 3.1. into *S. japonicus* vectors constructed in Task 2.2

Results: As episomal vectors for *S. japonicus* transformation are still not available, we amplified the *S. japonicus* and *C . albicans* genes and inserted them into expression cassettes constructed in work task 2.2. These constructs were then used in work tasks 3.2 and 3.3.

2.6. Transformation of *S. japonicus* mutants with expression vectors containing foreign genes. Evaluation of the phenotypes. In case of functional homology, the foreign gene suppresses the mutant phenotype.

Results: We managed to inactivate the *ace2Sj, agn1Sj, eng1Sj* and *sep1Sj* genes in *S. japonicus* (see above). All mutants were viable and showed at least mild cell separation

defects in the yeast phase. Because of the incomplete septum cleavage, the mutants formed chains of 3-15 non-separated yeast cells. These chains were not hyphae, because their cells did not switch to unicellular growth and did not form large vacuoles at their non-growing ends (essential attributes of the hyphal phase). When we transformed the mutants with expression cassettes carrying the wild-type *S. pombe* genes or the wild-type *C. albicans eng1* gene, the normal yeast morphology was restored at least partially. Thus, despite the differences in the C-terminal and N-terminal regions, the foreign proteins could substitute the *S. japonicus* genes (manuscript in preparation).

2.7. Measuring gene expression in wild-type, mutant and transformed *S. japonicus* cells by RT-PCR. In case of mutants of regulators (e.g. *sep1, ace2*), the putative target genes will also be involved in the activity tests.

Results: The RT-PCR experiments confirmed that the foreign genes were transcribed in the transformed *S. japonicus* mutants. The target genes of the Sep1p and Ace2p transcription factors (*eng1* and *agn1*) were silent in the mutants and active in the *sep1*- and *ace2*-transformants (manuscript in preparation).

3. Verification of functional homology of genes identified by in-silico analysis as putative orthologues of the *S. pombe* cell separation genes

3.1. Amplification of the coding sequences of the putative orthologues from the other species (*S. japonicus*, *C. albicans, Saccharomyces cerevisiae, S. octosporus*, etc.) and cloning them in *Escherichia coli*

Results : We cloned the coding regions of all cell separation genes from *S. japonicus* and *S. octosporus*. From *S. cerevisiae* and *C. albicans* we cloned the orthologues of *eng1*. For the amplification of the genes, we used primer pairs which were complementary to the beginning and the end of the coding regions and contained extensions for recognition sites of restriction endonucleases that also cleave the polylinker behind the thiamin-repressible *nmt* promoters of the pRep plasmids routinely used for heterologous expression of foreign genes in *S. pombe*. (See references 1, 5 and 6).

3.2. Integration of genes cloned in Task 3.1. into expression vectors with thiamin-repressible *nmt* promoters

Results: The cloned genes were inserted in three types of *S. pombe* expression vectors behind their *nmt* promoters. We constructed in this way plasmids from which the integrated genes could be transcribed with various efficiencies (strength). The *S. pombe* ace2$^-$, sep1$^-$, agn1$^-$ and eng1$^-$ mutants were then transformed with these plasmids. Transformants were selected on thiamin-rich medium to prevent the transcription of the *S. japonicus* genes. Then the transformants were shifted to a thiamin-free medium, on which the foreign genes could be transcribed. (See references 1, 5 and 6).

3.3. Transformation of *S. pombe* cell-separation mutants with the constructs and examination of the phenotypes of the transformants at various levels of heterologous gene expression. In case of functional homology, the foreign gene suppresses the mutant phenotype.

Results: By shifting the transformants to the thiamin-free medium, the mutant phenotypes were partially suppressed. The most dramatic rescue effects were observed in the ace2$^-$ and sep1$^-$ cells. Their cell-separation defects were almost completely suppressed by the medium-strength constructs, and the cells transformed with the strongest constructs showed phenotypes (swelling and lysis) that indicated over-activation of cell-separation genes regulated by the Sep1p-Ace2p cascade . The defects of agn1- and eng1- cells were only partially rescued when transformed with plasmids expressing the corresponding *S. japonicus* genes. (See references 1, 5 and 6).

3.4. In transformants without complementation phenotype: monitoring the transcription of the foreign genes by RT-PCR in the transformants under switch-on and switch-off conditions. If the gene is transcribed, but no complementation takes place, the gene

product is likely inactive in *S. pombe*.
Results: As the defects of the mutants were at least partially rescued by the heterologously expressed foreign genes, these tests were omitted.

References (papers describing the results of the project):

1. Balazs, A., Batta, G., Miklos, I., Acs-Szabo, L., Vazquez de Aldana, C.R., Sipiczki, M.: Conserved regulators of the cell separation process in *Schizosaccharomyces.* Fungal Genet. Biol. **49**:235-249, 2012
2. Papp, L., Sipiczki, M., Holb, I.J., Miklós, I.: Optimal conditions for mycelial growth of *Schizosaccharomyces japonicus* cells in liquid medium: it enables the molecular investigation of dimorphism. Yeast **31**:475-482, 2014
3. Munoz, J., Cortes, C.G., Sipiczki, M., Ramos, M., Clemente-Ramos, J.A., M. Moreno, M.B., Martins, I.M., Perez, P., Ribas, J.C.: Extracellular cell wall β(1,3)glucan is required to couple septation to actomyosin ring contraction. J. Cell Biol., **203**: 265-282, 2013
4. Nagy, L., Ohm, R., Kovacs, G., Floudas, D., Riley, R., Gacser, A., Sipiczki, M., Davis, J., Doty, S., De Hoog, G.S., Lang, B.F., Spatafora, J., Martin, F., Grigoriev, I., Hibbett, D.: Latent homology and convergent regulatory evolution underlies the repeated emergence of yeasts. Nature Communications **5**:4471, 2014
5. Sipiczki, M., Balazs, A.: Structure and phylogenesis of the cell-separation beta-glucanase Eng1p in Ascomycota. Submitted for publication, 2015
6. Sipiczki, M., Balazs, A., Monus, A., Papp, L., Horvath, A., Sveiczer, A., Miklos I.: Phylogenetic and comparative functional analysis of the cell-separation alpha-glucanase Agn1p in *Schizosaccharomyces*. Microbiology SGM **160**:1063-1074, 2014
7. Sipiczki, M.: Visualization of fission yeast cells by transmission electron microscopy. Methods Mol. Biol. **1369**:97-111, 2016