Gene expression during filamentous growth, in a non-pathogenic dimorphic haploid yeast, *Sch. japonicus.*

Final report

Background and aims:

Yeasts are members of normal flora of the skin, the gastrointestinal tract and the genitals. Generally, they are harmless. However, under special conditions these „harmless" cells are able to cause diseases. Namely, an important feature of these pathogenic yeasts is their ability to switch between different morphological forms (called dimorphism), which is thought to be critical for their pathogenesis.

Despite the intensive study of dimorphism, the mechanisms governing these morphogenetic conversions and the dimorphic genes are still not fully understood. Thus, in this project, effects of the environmental factors on the hyphae production, genetic background and signal transducing pathway of dimorphism were investigated in the fission yeast *Schizosaccharomyces japonicus*.

Study of relationship between environmental conditions and dimorphism
Optimisation of the RNA isolation method from hyphae
1st year

RNA sequencing in order to identify the genes involved in mycelial growth
Bioinformatic analyses of RNA sequencing data
2nd year

Study of regulators which are involved in the mycelial growth (pka1, fhl1)
Investigation of functional homology (rsv1)
3rd-4th years

Workflow of the project

Results:

Work plan 1.1. As dimorphism is connected to precise environmental sensing, we wanted to find those environmental factors which can influence hyphal production. Thus, different environmental factors were tested both in solid and liquid media.

Most important results: Our experiments revealed that lower external pH, higher temperature, and presence of pepton or CO_2 pressure were favourable to production of hyphae.

Furthermore, we managed to prove that in contrast to earlier results, cells of *S. japonicus* could produce hyphae in liquid medium, but they need special circumstances (inducers). These results are important because they can facilitate the molecular investigation of hyphae production.

The inducer agents were also identified. Interestingly, they were partly similar to the inducers of the distantly related *C. albicans* and other pathogenic yeasts (Barlow et al. 1974). Namely, FBS (Fetal Bovin Serum) and osmotic stress could induce strong mycelial growth in liquid medium. Furthermore, the inducer effects of FBS were concentration-, temperature- and pH dependent, similarly to *C. albicans* cells. This suggests that there must be general and common features of mycelial growth, even in distantly related species.

Work plan 1.2. To get more information about the relationship between environmental conditions and dimorphism and to find activators and inhibitory factors of mycelial growth, amino acids, nucleobases and drugs were also tested.

Most important results: We managed to identify further activators, such as methionine or inhibitors, such as proline, alcohol, or caffeine, which can be used to influence mycelial growth. These materials, especially the inhibitors, can later become potential antifungal agents and can play an important role in the protection against fungal infections.

The above results were published in: Papp L., Sipiczki M., Holb I., Miklós I. (2014) Optimal conditions for mycelial growth of Schizosaccharomyces japonicus cells in liquid medium: it enables the molecular investigation of dimorphism. YEAST, 31(12): 475-482

Work plan 1.3. Optimisation of the RNA isolation method from hyphae was important because the subsequent transcriptional profiling experiments required high-quality RNAs. Furthermore, we noticed that isolation of high-quality RNA was more difficult from hyphae than from yeast cells, as the hyphae contained large vacuoles and enzymes, which ruined the quality of the RNA. Thus, modification of the RNA isolation method (described by Lyne in 2003) was necessary.

Most important results: In order to isolate high-quality RNA, we tried to modify both the culturing conditions and the purification of RNA. In the end, elimination of vacuoles and RNA isolation from the apical parts of the hyphae, combined with repeated washing of the samples proved to be the best method.

Work plan 2.1. RNAs were isolated for the subsequent transcriptional profiling experiments using the modified method described in 1.3.
Most important results: High-quality RNAs were isolated from the wild-type yeast cells and hyphae.

Work plan 2.2. Originally RNA isolation from *sep1* mutant was also planned, because we assumed that the Sep1 transcription factor could be an important regulator of dimorphism. Namely, Sep1 regulates the cytokinesis of *S. pombe* (Ribar et al. 1997, Grallert et al.1999.) and inactivation of the *sep1* gene in the *S. japonicus* abolished cell separation and hampered hyphal fragmentation (Balazs et al. 2012).

At the same time, our results obtained in the RNA sequencing of wild-type hyphae showed that the *sep1* gene did not play a role in mycelial growth (see later in 2.3). This was a new and unexpected result. Thus, carrying out the RNS sequencing of *sep1* mutant strain was not necessary.

Work plan 2.3. RNA sequencing was carried out using the RNA samples isolated in 2.1. (Three separate experiments were carried out).
Most important results: Genes involved in the mycelial growth of *S. japonicus* were identified.

Work plan 2.4.Computer analyses of the RNA sequencing data were carried out. Predicted functions of the genes and their chromosomal localisations were determined in the Database of Broad Institute. GO (Gene Ontology) numbers of the genes were also identified using their orthologues in the *S. pombe* database (http://www.pombase.org). *The S. japonicus* database did not contain GO categories).
Most important results: Genes with different functions, such as transcriptional regulators, cell wall genes, cell separation genes, transporters etc. were identified. These results show that the yeast-to-hyphae morphological shift and hyphae elongation are very complex processes, which require a wide variety of gene-sets.

Work plan 2.5. Data of transcriptional profiling were compared with the results of microarray analyses originated from *Candida albicans* (Nantel et al. 2002, Biswas et al. 2007).

Most important results: Comparison revealed that there are common, probably evolutionarily conserved genes which are involved in the mycelial growth of both species. The most important common genes and their signalling pathway are shown in Fig.1. These results were in good agreement with our conclusions described in 1.1.

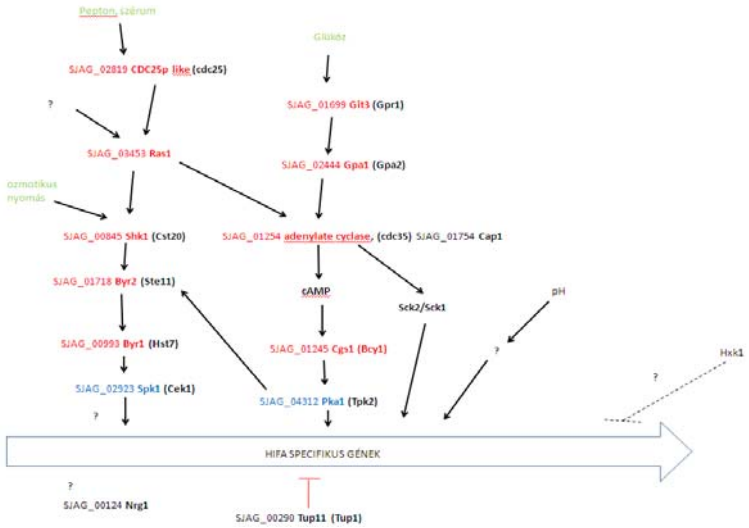

Fig.1. Regulation of dimorphism. Common genes in *S. japonicus* (e.g.SJAG_02819) and *C. albicans* (e.g.cdc25).

The manuscript is in the process of being prepared. Its possible title: Transcription profiling reveals genes involved in the mycelial growth of *Schizosaccharomyces japonicus*

Work plan 3.1-3.4. Genome-wide gene expression profiling revealed genes which are involved in the mycelial growth of *S. japonicus* ("target" genes)(described in 2.1-2.4.). Since several regulator genes (e.g. transcriptional regulators, kinases) were also found among the targets, it could be assumed that these regulators act on further genes and regulate defined groups of the dimorphic genes. To reveal the exact roles of these regulators in dimorphism and identify their target genes, two regulators (*pka1* and *fhl1*) were further analysed.

Most important results: *pka1* gene was amplified by PCR (Work plan 3.1), and was cloned into a cloning vector (Work plan 3.2). The gene was disrupted with KanMX cassette (Work plan 3.3.), and this DNA construction was introduced into the *S. japonicus* cells with integrative transformation (Work plan 3.4). These experiments enabled us to isolate *pka1*Δ mutant strain.

At the same time, these experiments also shed light on the fact that *S. japonicus* cells are hard to transform and they probably have a very strong non-homologous recombination system,

unlike the closely related *S. pombe*. It was a new and unexpected result, which can suggest that *S. japonicus* preserved more ancient features of its filamentous ancestors than the *S. pombe*.

Work plan 3.5. Phenotypic analysis and microscopic examination of the *pka1* deleted cells were carried out.

Most important results: Pka1 is involved in mycelial growth, as the mutant produced shorter hyphae than the wild-type strain. This gene can be an evolutionarily conserved member of dimorphism, as its homologous genes in *S. cerevisiae* and *C. albicans* (TPK1-3 genes) also regulate mycelial growth (Bockmühl et al. 2001, Giacometti et al. 2011). It was also clearly visible that the true hyphae of the *pka1*Δ preserved strong vacuolization, similarly to the wild-type hyphae.

Our tests also shed light on the fact that poor nutritional conditions and CO_2 pressure strongly inhibited mycelial growth of the *pka1*Δ, which could slightly be overridden by a higher amount of vitamin. Since their morphological transition could happen even under these special conditions (poor medium, CO_2 pressure) we supposed that it was the elongation of hyphae not the switch that was inhibited in the *pka1* mutant.

Work plan 3.6. In this part of the project we wanted to get information about the genes and processes which are regulated by Pka1. Since identification and investigation of the proteins seemed to be more slow and expensive than the global analysis of the transcriptional network, thus RNA sequencing analysis of the mutant strain was carried out. The steps and the methods were similar to the ones used previously.

Most important results: The analysis revealed that 373 genes and ORFs were up- or down-regulated greater than twofold in the *pka1*Δ cells compared to the wild-type strain.

pka1 is a multifunctional gene, as it regulates different cell processes, such as translation, modification of proteins, meiosis, mitochondrion organization, oxidative stress, aging and ribosome biogenesis as well as dimorphic genes.

In good agreement with our earlier results, the putative homologues of known *Candida* filament-associated genes were also found among the targets. Thus we can presume that these genes can be the common and evolutionarily conserved participants of dimorphism.

Comparison of the *pka1* targets with the dimorphic genes described in 2.4. enabled us to identify the *pka1* regulated sub-cluster of the genes involved in mycelial growth.

Further comparison of the *pka1* targets with the *pka1* regulated genes of *S. pombe* revealed that a significant part of the targets could be evolutionarily conserved within the *Schizosaccharomyces* genus (Liu et al. 2015).

The above results were published in: László Papp · Matthias Sipiczki · Ida Miklós: Expression pattern and phenotypic characterization of the mutant strain reveals target genes and processes regulated by pka1 in the dimorphic fission yeast Schizosaccharomyces japonicus, Curr Genet, DOI 10.1007/s00294-016-0651-x

Investigation of the second dimorphic gene:

From the RNA sequencing data (described in 2.3.) a further regulator gene, whose *Candida* homologue also plays a role in dimorphism, was chosen for the next gene deletion. The experiments were launched, but the integrative transformations resulted in only inappropriate (non-homologous) transformant cells and this hampered the successful creation of the second gene-deletion.

In consequence, instead of the gene being deleted, its *S. pombe* homologue was investigated. Namely, the *fhl1* deleted strain was available as it was earlier isolated in our laboratory (Szilagyi et al. 2005).

Most important results: Genetic and molecular analysis of the *fhl1* fork-head type transcription factor revealed that it regulates 72 genes on minimal and 75 genes on complete medium. These experiments also revealed that target genes of the same transcriptional regulator can be partly different depending on environmental conditions. However, most of the genes could be classified into the same GO categories.

Several target genes of the *fhl1,* such as *mei2, ste4* were involved in mating and sporulation. This correlated well with results that the *fhl1* mutant was sensitive to the concentration of nitrogen in the media and showed higher mating efficiency than the wild-type strain. Another group of genes that is altered by loss of *fhl1+* function was classified as transporters and permeases.

Since nitrogen starvation response is controlled by *tor2* (Matsuo et al. 2007), the target genes of *fhl1* and *tor2* were also compared. A significant degree of overlap was found between *tor2* and *fhl1* fork-head transcription factor in the regulation of these genes.

In order to prove relation of *fhl1* and *tor2*, the *fhl1* gene was cloned into an expression vector (Maundrell 1993) and this DNA construction was introduced into the *tor2* mutant cells. Since the expression of *fhl1* from a strong promoter could rescue rapamycin and temperature sensitivity and suppressed the hyper-sporulation defect of the *tor2-ts* mutant cells, we supposed that Fhl1 acts in the TOR signalling pathway, downstream of Tor2.

*The above results were published in: Emese Pataki, Ronit Weisman, Matthias Sipiczki, Ida Miklos: fhl1 gene of the fission yeast regulates transcription of meiotic genes and nitrogen starvation response, downstream of the TORC1 pathway.Current Genetics,*CUGE-D-16-00038R1

Investigation of functional homology

As our earlier results had suggested that evolutionarily conserved genes can function in the regulation of environmental sensing and mycelial growth, we tried to experimentally prove the functional homology of a regulator gene. The zinc-finger transcription factor (*rsv1*) and its homologues were investigated using bioinformatic methods and interspecific complementation analysis.

Most important results:

The homologous genes of *rsv1* were identified by reciprocal best-hit Blastp searches in the *Schizosaccharomyces* and *Candida albicans* species (*rsv1* genes of *S. japonicus* and *S. octosporus*, and *Cas5* gene of *C. albicans)*, and their sequence analyses were carried out.

Later, *rsv1* genes of *S. japonicus* and *S. octosporus*, and *Cas5* gene of *C. albicans* were cloned into the pREP41 vector (Maundrell 1993) and Rsv1/Cas5 proteins were expressed from a quite strong promoter in the *S. pombe rsv1* mutant cells. Since the homologous transcription factors could restore the stress sensitivity of the *S. pombe rsv1* mutant, we managed to prove functional homology of the homologous genes and identify a new conserved regulator of the genus.

The manuscript is in the process of reviewing. Its possible title: Schizosaccharomyces pombe rsv1 transcription factor and its putative homologues preserved their functional homology and are evolutionarily conserved

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Use of the results:

Our results revealed the genes which are involved in the mycelial growth of *S. japonicus,* together with their functions and chromosomal localisations. The *pka1* regulated sub-cluster of the dimorphic genes was also identified with the evolutionarily conserved members of the filament associated signalling pathway. Several aspects of the relationship between dimorphism and environmental conditions were also analysed.

This was the first attempt to identify the genetic background of yeast–to hypha transition in *S. japonicus.* Our data can contribute to a better understanding of the molecular mechanism of dimorphism and virulence of pathogenic fungi. The results enable us to find new potential anti-mycelial drugs or new drug “targets”.

Discrepancy:

Cause of the time slip: Isolation of the integrative transformant cells was harder and took longer time than we expected. It revealed that *S. japonicus* has a very strong non-homologous recombination system compared with the *S. pombe* cells. Thus the *pka1* deleted strain was isolated later in time than planned.

Unsuccessful deletion of the *S. japonicus fhl1* gene was also related to the problem mentioned above. That is why the *S. pombe* homologous gene (*fhl1*) was investigated instead of *S. japonicus fhl1*.

Work plan 2.2. RNA seq. data of the wild-type hyphae (2.3) revealed that the *sep1* gene does not play an important role in mycelial growth. This was a new and unexpected result. Consequently, it became unnecessary to carry out the RNS sequencing of *sep1* mutant strain.

Work plan 2.3. Change in the method. We used RNA sequencing instead of microarray analysis to find the genes which are involved in mycelial growth. We preferred RNA sequencing, as the microarray chips were not available in *S. japonicus* and it would have been more expensive to produce them.

Work plan 3.6. Change in the method. In this part of the project we wanted to get information about the genes and processes which are regulated by Pka1. Since identification and investigation of the proteins seemed to be slower and more expensive than the global analysis of the transcriptional network, RNA sequencing analysis of the mutant strain was carried out.

Plus results: The work plan did not include investigation of the functional homology of a regulator gene. As several results suggested that certain regulator genes of dimorphism can be evolutionarily conserved, we thought that it was worth investigating and proving it experimentally.

Publications:

1. Papp L., Sipiczki M., Holb I., Miklós I. (2014) Optimal conditions for mycelial growth of Schizosaccharomyces japonicus cells in liquid medium: it enables the molecular investigation of dimorphism. ***YEAST****, 31(12): 475-482*

2.László Papp · Matthias Sipiczki · Ida Miklós: Expression pattern and phenotypic characterization of the mutant strain reveals target genes and processes regulated by pka1 in the

dimorphic fission yeast Schizosaccharomyces japonicus, ***Current Genetics,*** *DOI 10.1007/s00294-016-0651-x*

*3.The above results were published in: Emese Pataki, Ronit Weisman, Matthias Sipiczki, Ida Miklos: fhl1 gene of the fission yeast regulates transcription of meiotic genes and nitrogen starvation response, downstream of the TORC1 pathway.****Current Genetics****,*CUGE-D-16-00038R1

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