Final report

Carbon stress response

Carbon stress responses of *Aspergillus nidulans* were recorded by next generation RNA-sequencing (RNAseq) on lactose and on arabinogalactan (carbon limitation stresses) as well as under carbon starving conditions using reference cultures growing on glucose. The transcription of approximately 3000-4000 genes in each case was significantly depended on the applied stress. Among them 1192 genes showed up-regulation and 921 genes showed down-regulation during all the three carbon stress treatments. Up-regulation of secondary metabolite cluster genes and genes encoding carbohydrate-active enzymes (CAZymes) as well as down-regulation of genes involved in glucose utilization, respiration, and oxidative phosphorylation were observed in each stress treatment. Up-regulation of autophagy-related genes, genes encoding hemicellulose and pectin hydrolyzing enzymes and of D-galactose oxidoreductive pathway genes were characteristic for carbon starved, arabinogalactan and lactose containing cultures, respectively. Interestingly, up-regulation of autolytic cell wall degradation genes were observed both in carbon starved and arabinogalactan containing cultures. Meanwhile, up-regulation of lactose permease and β-galactosidase genes (including *lacpA*, *lacpB,* and *lacD*) were observed in all the three carbon stressed cultures. Our results support the view that adaptation to carbon stress starts with the secretion of various hydrolyzing enzymes. Later, the compounds liberated by these enzymes can up-regulate further genes needed for the utilization of the "discovered" nutrients. This mechanism explains why the early stress responses to different carbon stresses are very similar and also elucidates how fungi can easily adapt to grow on special carbon sources like lactose (1-3).

A. nidulans gamma-glutamyl transpeptidase was partially purified from the fermentation broth of carbon stressed cultures. The enzyme showed only a small hydrolase activity. It utilized Gln, glutathione and less efficiently oxidized glutathione as gamma-glutamyl donors and amino-acids and peptides (including Glu, Cys, Met, Gly-Gly and Cys-Gly) but not hydroxylamine as gamma-glutamyl acceptors. We propose that the function of this enzyme is not to degrade glutathione and other gamma-glutamyl compounds but to produce them. This enzyme may contribute to the utilization of extracellular peptides and amino-acids in carbon stressed cultures (4).

Double deletion of *chiB* and *engA* (encoding an extracellular endochitinase and a β-1,3-endoglucanase, respectively) decreased conidia production under carbon-stressed conditions, suggesting that these autolytic hydrolases can support conidia formation by releasing nutrients from the cell wall polysaccharides of dead hyphae. Double deletion of *prtA* and *pepJ* (both genes encode extracellular proteases) reduced the number of cleistothecia even under carbon-rich conditions except in the presence of casamino acids, which supports the view that sexual development and amino acid metabolism are tightly connected to each other in this fungus (5).

Melanin production showed strong positive correlation with the activity of the secreted chitinase and ß-1,3-glucanase. Deletion of either *chiB* or *engA* or both, almost completely prevented melanization of carbon stressed cultures. In contrast, addition of *Trichoderma* lyticase to cultures induced melanin production. Synthetic melanin could efficiently inhibit the purified ChiB chitinase activity. It could also efficiently decrease the intensity of hyphal fragmentation and pellet disorganization in *Trichoderma* lyticase treated cultures. Glyphosate, an inhibitor of L-3,4-dihydroxyphenylalaninetype melanin synthesis, could prevent melanization of carbon-starved cultures and enhanced pellet disorganization, while pyroquilon, a 1,8-dihydroxynaphthalene-type melanin synthesis inhibitor, enhanced melanization, and prevented pellet disorganization. We concluded that cell wall stress induced by autolytic cell

wall hydrolases was responsible for melanization of carbon-starved cultures. The produced melanin can shield the living cells but may not inhibit the degradation and reutilization of cell wall materials of dead hyphae. Controlling the activity of autolytic hydrolase production can be an efficient approach to prevent unwanted melanization in the fermentation industry, while applying melanin synthesis inhibitors can decrease the resistance of pathogenic fungi against the chitinases produced by the host organism (6).

Deletion of *dugB*, *dugC* or both genes in *A. nidulans* resulted in a moderate increase in glutathione content under growing conditions and substantially slowed down the depletion of glutathione pools under carbon starvation. Inactivation of dug genes also caused reduced accumulation of reactive oxygen species, decreased autolytic cell wall degradation and enzyme secretion but increased sterigmatocystin formation in starving cultures. Changes in the transcriptomes suggested that enzyme secretions were controlled at post transcriptional level. In contrast, secondary metabolite production was also regulated at the level of mRNA abundance. We suggest that glutathione connects starvation and redox regulation to each other: Cells utilize glutathione as stored carbon source during starvation. The reduction of glutathione content alters the redox state activating regulatory pathways responsible for carbon starvation stress responses. Under glucose rich conditions, inactivation of dug genes reduced conidia production, disturbed sexual development and down-regulated the transcription of genes encoding MAP kinase pathway proteins or proteins involved in the regulation of conidiogenesis or sexual differentiation. These findings indicate that the authority of redox regulation goes far beyond the protection against redox stress; it affects development, stress responses and secondary metabolism as well (7-8).

Carbon stress responses of *Aspergillus fumigatus* were recorded by RNAseq on casein pepton as well as under carbon starving conditions using reference cultures growing on glucose or on glucose and casein pepton. The presence of glucose substantially affected the transcriptome both in the presence or absence of casein pepton. The number of differentially expressed genes were more than 4000 in both cases. The presence of pepton had stronger effect on the genome wide transcription of *A. fumigatus* in glucose free media (the number of differentially expressed genes were more than 2000) than in the presence of glucose (the number of differentially expressed genes were less than 1000). Interestingly, the transcription of gliotoxin and fumagillin cluster genes showed the highest activity in glucose containing but pepton free media.

Combinatorial stress response

Combinatorial stress responses were studied with *A. nidulans* (menadione induced oxidative stress response under carbon starvation stress) and with *A. fumigatus* (hydrogen-peroxide induced oxidative stress response under iron starvation stress).
We found the followings:

A. nidulans cultures growing on glucose were more sensitive to menadione, *terc*-butyl-hydroperoxide or hydrogen-peroxide than carbon starving cultures. Moreover, although carbon starvation itself increased the reactive oxygen species (ROS) content of cells, menadion treatment caused significantly higher increase in ROS levels on glucose than under carbon starvation. Transcriptom (RNAseq) data also demonstrated that growing cultures were affected by menadione stress more intensively than starving cultures. Carbon starvation up-regulated several genes encoding antioxidative enzymes which can explain the increased oxidative stress tolerance of these cultures. Menadione treatment down-regulated the transcription of genes encoding extracellular proteins on glucose, while carbon starvation up-regulated most of them. Combination of the two treatments resulted in either up-regulation or down-regulation of these genes. The gene encoding the AtfA transcription factor was down-regulated under carbon

starvation stress and also under the combined oxidative – carbon starvation stress. It suggests that AtfA should have only a minor role in the regulation of oxidative stress response in starving cultures. In contrast, the *zipB* genes showed strong up-regulation under the previously mentioned conditions. To demonstrate the importance of ZipB in the regulation of oxidative stress response *delta-zipB* strains were created. The surface culture experiments with these strains demonstrated that ZipB was important in the menadione stress tolerance under carbon limited conditions (9-10).

Both RNAseq and physiological experiments demonstrated the existence of synergistic interaction between iron starvation and oxidative stress in *A. fumigatus*. This phenomenon, if generalized to other microbes, may explain why limitation of iron access together with an oxidative attack on pathogens is a widespread strategy to combat infections in both plants and animals. The stress response to oxidative stress combined with iron starvation differed in both strength and character from either oxidative stress response or iron starvation stress response. It means that we have to be very careful if we want to predict the *in vivo* behavior of microbes based on data obtained from *in vitro* studies. Even a relatively small difference between the *in vitro* and *in vivo* systems can substantially modify the (stress) response of the microbes. During the combined (iron starvation plus oxidative stress) stress treatment, the following changes were observed: Heat shock response, DNA repair, and oxidative stress response genes were upregulated which was followed by the upregulation of genes included in macroautophagy and ubiquitin-dependent protein degradation. The latter presumably not only made it possible to eliminate the damaged proteins, but also increased the amount of (re)usable iron by degrading the damaged or excess iron-containing proteins. Transcription of genes involved in ribosome biogenesis and translation decreased, despite the fact that iron starvation alone led to their significant down-regulation. These changes may have contributed to further decrease of the growth and thus the iron demand of cultures. Although iron-dependent processes were generally down-regulated some genes encoding iron-containing proteins (i.e. heme and Fe-S cluster binding proteins) as well as genes involved in Fe-S cluster biosynthesis showed significant up-regulation. Based on the proteomic studies, the upregulation of these genes, in the absence of sufficient iron, was not able to increase the amount of the proteins. However, these changes may have contributed significantly to ensuring that the amount of iron-containing proteins, which is essential for survival, will not decrease to very low levels. Based on this, inhibition of iron-containing protein formation can be a potential therapeutic target in the future. The combined stress also up-regulated the transcription of numerous genes encoding multidrug transporters thus it may have affected the sensitivity of the fungus to antifungal agents. Moreover, an increase in the abundance of the AbcB protein responsible for cyp51a-independent azole resistance was also detected. These results further emphasize the relevance of the inhibition of efflux systems during antifungal therapy (11).

Involvement of *A. nidulans* AtfA in the regulation of stress responses

Although AtfA did not seem to be important in the regulation of either carbon stress response or oxidative stress response of carbon stressed cultures it affected the stress responses of cultures growing on glucose.

Deletion of *atfA* increased the oxidative stress sensitivity of *A. nidulans* and affected mRNA accumulation of several genes under both unstressed and stressed conditions. Both oxidative and salt stresses induced expression of some secondary metabolite gene clusters and the deletion of atfA enhanced the stress responsiveness of additional clusters. Moreover, certain clusters were down-regulated by the stresses tested (12-13). Several elements of oxidative stress response, including the down-regulation of the mitotic cell cycle, the menadione stress-specific up-regulation of Fe-S cluster assembly and the menadione stress-specific down-regulation of nitrate reduction, tricarboxylic acid cycle and ER to Golgi vesicle-mediated transport showed

AtfA dependence (13-14). To elucidate the potential global regulatory role of AtfA governing expression of a high number of genes with very versatile biological functions, we devised a model based on the comprehensive transcriptomic data. Our model suggests that an important function of AtfA is to modulate the transduction of stress signals. Although it may regulate directly only a limited number of genes, these include elements of the signaling network, for example, members of the two-component signal transduction systems. AtfA acts in a stress-specific manner, which may further increase the number and diversity of AtfA-dependent genes (14). Comparing the differences of the transcriptomes of the wild type and the *delta-atfA* strains under ten different culturing conditions resulted in a set of genes showing consistent down-regulation in the mutant in comparison to the reference strain. This gene set was enriched in genes encoding elements of the two-component signal transduction. The promoter of most of these genes contained one or even more putative AtfA binding sites. It supports our view that the main function of AtfA is modulating the activity of the signaling network under stress (15). Studying of stress responses induced by Cd stress in the delta-atfA and the reference strains demonstrates that deletion of a gene encoding a regulatory protein can substantially alter the stress response even if it does not influence the stress tolerance of the strain (16-18).

Results with the theoretical background of stress responses

We performed a large-scale homology analysis of stress proteins and generated and analyzed three stress defense system models based on *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans*. Although both yeast-based and *A. nidulans*-based models were suitable to trace evolutionary changes, the *A. nidulans*-based model performed better in mapping stress protein radiations. The strong Mantel correlation found between the positions of species in the phylogenetic tree on the one hand and either in the *A. nidulans*-based or *S. cerevisiae*-based models on the other hand demonstrated that stress protein expansions and reductions contributed significantly to the evolution of the *Aspergilli*. Interestingly, stress tolerance attributes correlated well with the number of orthologs only for a few stress proteins. For most stress proteins, changes in the number of orthologs did not correlate well with any stress tolerance attributes. As a consequence, stress tolerance patterns of the studied *Aspergilli* did not correlate with either the sets of stress response proteins in general or with the phylogeny of the species studied. These observations suggest that there are other processes, which may counterbalance the effects of stress gene duplications or deletions including (i) alterations in the structures of stress proteins leading to changes in their biological activities, (ii) varying biosynthesis of stress proteins, (iii) rewiring stress response regulatory networks or even (iv) acquiring new stress response genes by horizontal gene transfer. All these multilevel changes are indispensable for the successful adaptation of filamentous fungi to altering environmental conditions, especially when these organisms are entering new ecological niches (19-20).

Stress responses recorded by transcriptome analyses represent a difference between untreated and treated cultures. As a consequence, it equally shows how cells try to adapt to the new conditions and how the adaptation to the old conditions is ceasing. Therefore, if the unstressed conditions are unique (like the glucose rich, fast growing cultures) the latter can dominate the stress response (15).

Summary of related publications

Papers published in peer reviewed journals: 13

Further papers: 1 under revision ("DUG pathway governs degradation of intracellular glutathione in *Aspergillus nidulans*"), 1 submitted ("AtfA is not essential for the efficient

adaptation to $CdCl_2$ stress, yet deletion of the *atfA* gene alters markedly the stress response in *Aspergillus nidulans*") and 3 under preparation ("Carbon limitation stress responses in *Aspergillus nidulans*", "Oxidative stress response of *Aspergillus nidulans* under carbon stress" and "Carbon stress in *Aspergillus fumigatus*")

Conference abstracts: 5 (Hungarian) and 14 (International)

Diploma works: 6 (MSc) and 7 (BSc)
PhD thesis: 1 (Zsolt Sptzmüller) and another one is in progress (Vivien Kurucz)
DSc thesis: 1 (Tamás Emri)

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