SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Regulation of glutathione metabolism, enzyme secretion and secondary metabolite production in *Aspergillus nidulans* under carbon stress

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Abbreviations

CAZyme: carbohydrate-active enzyme

CSR: carbon-stress-response

DCF: 2,7-dichlorofluorescein

DCM: dry cell mass

DTT: ditiotreitol

DUG: deficient in utilization of glutathione phenotype

ER: endoplasmic reticulum

ESR: environmental stress response

GSE: gene set enrichment

GSH: reduced glutathione

GSSG: oxidised glutathione

γGT: γ-glutamyl transpeptidase

MAPK: mitogen-activated protein kinase

MTT: methyltriazole tetrazolium

RNAseq: RNA sequencing

ROS: reactive oxygen species

RT-qPCR: quantitative reverse transcription polymerase chain reaction

SMG: secondary metabolite gene

SOD: superoxide dismutase

STC: sterigmatocystin

1. Introduction and objective

1.1. Introduction

In *Saccharomyces cerevisiae* and *Candida albicans*, it has been shown that proteins of the so-called DUG ("deficient in utilization of glutathione") pathway (Dug1, Dug2, Dug3) are primarily involved in intracellular glutathione (GSH) degradation, but in *Aspergillus nidulans*, genes of these proteins (*dugA/AN3459*: orthologue to *S. cerevisiae dug1*; *dugB/AN1879*: orthologue to *S. cerevisiae dug2*; *dugC/AN1092*: orthologue to *S. cerevisiae dug3*) have not yet been elucidated (Spitzmüller et al., 2015a). It has been observed previously that the GSH content of hyphae in *Penicillium chrysogenum* and *A. nidulans* cultures is significantly reduced and γGT activity is induced under carbon starvation stress (Emri et al., 2004). However, it has also been found that deletion of *ggtA* (gene encoding γGT) has no significant effect on the decrease in GSH concentration during carbon starvation *in A.* nidulans (Spitzmüller et al., 2015b). In contrast, the transcriptional activity of *dugA* and *dugC* was positively correlated with GSH degradation, whereas no similar correlation was observed for the expression of *ggtA* and *dugB* (Spitzmüller et al., 2015a). The clarification of the degradation of GSH, as an antioxidant molecule is also justified from a practical point of view because fungi may encounter stress conditions (e.g. carbon stress and oxidative stress) not only in nature but also under industrial conditions that may stimulate the production of undesirable secondary metabolites (e.g. mycotoxins), which are considered a promising option for the reduction of antioxidant production (Koza et al., 2022).

Filamentous fungi in general are characterised by a very diverse enzyme secretion and secondary metabolite production. The enzymes and secondary metabolites they secrete largely determine the distribution of species (their ability to utilize biopolymers of a given habitat), their virulence (pathogenicity), as well as interspecific competition and inter/intraspecific cooperation (Keller, 2019; Sakekar et al, 2021). Enzymes involved in the biosynthesis, modification, binding and

degradation of various carbohydrates are called carbohydrate-active enzymes (CAZyme) (Davies et al., 2005). More recently, CAZymes have attracted attention because of their biotechnological and industrial applications, as their activity has made it possible to efficiently saccharify complex plant materials. The precursor materials thus generated can be used for the production of various bio-based products, such as food, animal feed, paper, textiles, or other compounds, including biofuels (Chettri et al., 2020).

In parallel with global climate change, mycotoxin-producing fungal species are becoming a growing problem, which also calls for research on secondary metabolite production by fungi. Hungary may be one of the most vulnerable countries in Europe to the consequences of climate change; the spread of warm-loving toxinogenic *Aspergillus* species, such as the aflatoxin-producing *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nominus*, which are currently only found in the south of Hungary, may become a serious problem from both an agricultural and a health point of view. Since their secondary metabolites can also significantly influence the virulence of human pathogenic fungi (e.g. *Aspergillus fumigatus*), the formation of these metabolites under stress is also clinically worthwhile to consider.

When the availability or quality of the carbon source is insufficient to sustain rapid vegetative growth, cells undergo carbon stress (carbon starvation and carbon limitation). Carbon stress significantly affects not only the GSH metabolism of fungi, but also the secondary metabolite and CAZyme production. Several phenomena of interest (activation of GSH degradation pathways, mycotoxin formation, production of certain CAZyme proteins) are easier to study on carbon starved or carbon source limited cultures than under glucose (Pócsi et al., 2003, 2004; Contesini et al., 2021). By monitoring transcriptome changes, we can obtain a comprehensive picture of changes in the behaviour of the culture as a whole. The focus of our experiments was the processing and interpretation of transcriptome data collected by RNA sequencing (RNAseq), which allowed us to interpret GSH metabolism, mycotoxins and CAZyme protein formation as part of the carbon stress response. It is hoped that this approach

will give us a more comprehensive picture of the processes than the study of a single enzyme or metabolite.

1.2. Objective

For my PhD thesis, I aimed to gain a better understanding of the carbon stress response of the model organism *A. nidulans*. In the first half of the thesis, I wanted to confirm the function of *dugB* and *dugC*, genes putatively involved in GSH degradation, and then to investigate the effects of GSH degradation inhibition on the fungal physiology, with particular emphasis on changes in CAZyme formation and secondary metabolism under carbon starved conditions and under glucose carbon source. In the second part of the thesis, I studied the carbon source limitation stress response observed on lactose carbon source and compared it with the changes observed on glucose, arabinogalactan and in the absence of carbon sources. I was primarily interested in answering the question of how the quality of the carbon source affects the CAZyme production and secondary metabolism of the fungus.

The following experiments were designed/performed:

1. Examination of GSH metabolism during carbon starvation

Comparison of *ΔdugB*, *ΔdugC* and *ΔdugB-ΔdugC* gene-deleted strains and a reference strain of *A. nidulans* in submerged (liquid) culture on carbon source-free (carbon starved cultures) and glucose-containing (growth cultures) media: biomass accumulation (DCM); determination of glutathione content changes (GSH, GSSG); redox status monitoring (DCF assay); determination of antioxidant and autolysis-related enzyme activities; comparison (RNAseq) and validation (RT-qPCR) of global gene expression patterns; analysis of secondary metabolite production (TLC, HPLC, gene cluster analysis).

Comparison of surface cultures (on agar plates) of the above *A. nidulans* strains on glucose-rich (40 g l^{-1}) and carbon source-limited (10 g l^{-1} glucose) media: tolerance of reductive, oxidative, osmotic, heavy metal and cell wall stress (determination of colony size); conidia and ascospore formation analysis.

2. Investigation of carbon limitation stress response under lactose

Four submerged *A.* nidulans cultures that contained glucose (as a readily utilizable monosaccharide), lactose (as a disaccharide that allows slower growth than glucose), arabinogalactan (as a complex polysaccharide that allows only slow growth), or no carbon source at all: Monitoring metabolic activity (MTT assay); biomass accumulation (DCM); determination of enzyme activities related to autolysis and lactose utilization; identification of extracellular proteins (2D-PAGE, nanoHPLC); comparison (RNAseq) and validation (RT-qPCR) of global gene expression patterns; investigation of secondary metabolite production (TLC, gene cluster analysis).

3. Materials and methods

The *A. nidulans* strains tested in our experiments were THS30 (reference strain), THM2 (*ΔdugB* mutant), THM3 (*ΔdugC* mutant), THM4 (*ΔdugB ΔdugC* mutant), THM7 (*dugC*$^{+}$ complemented strain), THM8 (*dugB*$^{+}$ complemented strain). The deletion and complemented strains were generated and provided by Dr. Heungyun Harrison Moon and Prof. Dr. Jae-Hyuk Yu (University of Wisconsin-Madison).

Strain maintenance and surface culture studies were carried out using Barratt's minimal nutrient agar (AMM) (Barratt et al, 1965). The media contained the stress agents separately before inoculation, at the following concentrations: 0.18 mM menadione sodium bisulfite (MSB); 0.8 mM *tert-butyl hydroperoxide* (*tBOOH*); 1.5 mM diamide; 1 M NaCl, 50 mM congo red (CR). To quantify the amount of conidia produced by surface cultures, the spore counts from the surface of unit agarose spores were determined using a hemocytometer (Emri et al., 2018).

For the submerged cultures, Barratt's medium (agar-free version of the medium) was used in flasks in a frosted Erlenmeyer flask. After rinsing the mycelia, which were still in the exponential growth phase, the carbon source content of the fresh broths was as follows: carbon source free (carbon starved strains), 20 g l^{-1} lactose (lactose/ carbon source limited strains), 20 g l^{-1} arabinogalactan (arabinogalactan/ carbon source limited strains), 20 g l^{-1} glucose (glucose/growing reference strains) (Gila et al., 2021, 2022).

RNA isolation was performed from mycelia filtered from fermented broth, from which RNA content was extracted using Chomczynski's method(1993). The RNA samples were checked and sequenced by the staff of the Laboratory of Genomic Medicine and Bioinformatics Core Facility, UD.

In RT-qPCR assays, the relative transcription rate was determined by the ΔΔCt method. The reference genes used were *gfdA* and AN6700, depending on the experimental series (Gila et al., 2021, 2022).

The RNA sequencing and the determination of differentially expressed genes was performed by Dr. Károly Antal (Eszterházy Károly Catholic University)

The determination of biological processes that can be linked to up-regulated or down-regulated genes was characterized using Gene Set Enrichment (GSE) analyses. Enrichment of genes belonging to the defined categories was tested using the Fisher exact test. GSE analyses were also performed for secondary metabolite cluster genes on a cluster-by-cluster basis (Gila et al., 2021, 2022).

For physiological studies, filtered mycelial samples were used. For intracellular parameters, samples were digested using a homogenizer. The growth of the submerged cultures was characterized by determining the dry cell mass (DCM). MTT assay was performed to determine the metabolic activity ('viability') of the cultures. The extent of carbon source utilisation was determined by quantifying the amount of reducing sugars in the fermentation broth. Changes in the redox balance of the cultures were investigated by DCF assay. Protein contents were also examined in the assayed cell-free mycelial extracts and filtered fermentation broths using the Bradford method (Gila et al., 2021, 2022).

The protein, glutathione (GSH, GSSG) and melanin contents were determined and enzyme activity (protease, chitinase, β-glucosidase, β-galactosidase, hexose aminidase, γ-GT, cellulase, catalase, nitrate reductase, glutathione reductase, SOD) was measured using a spectrophotometer (Szilágyi et al., 2018).

For qualitative control of STC production in the cultures, thin layer chromatography (TLC) was used (Ámon et al., 2018). The STC content of mycelia was quantified by HPLC analysis with the help of Prof. Dr. Tünde Pusztahelyi (Agricultural Instrumentation Centre, UD).

For the proteomic analysis of proteins secreted into the fermentation broth, the separation of the proteins by 2D gel electrophoresis was performed by Dr. Judit Keserű (Department of Human Genetics, UD). The identification of the proteins from the LC-MS/MS data was performed by Prof. Dr. Éva Csősz and colleagues (Institute of Biochemistry and Molecular Biology, UD).

4. Results

4.1 Examination of GSH metabolism during carbon starvation

Dr. Heungyun Harrison Moon and Prof. Dr. Jae-Hyuk Yu (University of Wisconsin-Madison, Madison, WI, USA) provided three *A. nidulans* strains (a *ΔdugB* and a *ΔdugC* mutant and *a ΔdugB-ΔdugC* double mutant) and their complemented strains to elucidate the role of the putative DugB-DugC complex in the cytosolic degradation of GSH. First, the expression activity of the *dugB* and *dugC* genes was investigated in carbon-starved cultures of mutant, reference and complemented strains. Interestingly, deletion of *dugC* also reduced the expression of *dugB*, whose activity was restored by complementation of *dugC*. In contrast, neither the absence of *dugB* nor its complementation affected the transcription of *dugC*. Gene deletions did not alter the GSSG content of cells.Under carbon-deprived conditions, all deletion strains had significantly higher GSH content. However, complementation of *the ΔdugB* and *ΔdugC* deletion strains with the *dugB* and *dugC* genes, respectively, reduced the GSH content of the strains and restored the phenotype of the reference strain THS30. The uniform increase in GSH content in the deletion mutants suggests that both DugB and DugC are involved in the degradation of cytosolic GSH during carbon starvation and that they are not able to substitute for each other. This is in agreement with the finding that Dug2p and Dug3p (orthologs to DugB and DugC in *S. cerevisiae*) form an $\alpha_2\beta_2$ heterotetramer in yeast cells, and only this heterotetramer has GSH-hydrolyzing (glutamine amidotransferase) activity. Thus, the degradation of GSH can be regulated by either *dugB* or *dugC* due to the heterotetrameric nature of the heterotetramer. The observed asymmetric interaction between the two genes (e.g. DugC can up-regulate *dugB*) may help to fine-tune the effects on the expression of *dugB* and *dugC*. This may be particularly important when opposing effects regulate the activity of the two genes.

In the submerged culture studies, exponential phase growing mycelial pellets were washed into carbon source-free culture medium. As expected, deletion of *dugB* and/or *dugC* resulted in elevated GSH levels compared to the control strain. There was

no difference in GSSG content of the mycelia in either case, and neither GSH nor GSSG was detected in the fermentation broth. Interestingly, even the double deletion of *dugB* and *dugC* did not completely block the depletion of GSH pools. These data suggest that pathway(s) other than the DUG pathway contribute to the degradation of GSH during carbon starvation.

The reduced GSH degradation was accompanied by a delayed upset of the redox balance, suggesting that reduced GSH levels during carbon starvation are partly responsible for the accumulation of reactive oxygen species. It is important to stress, however, that there was no significant difference in the redox status of the strains included in the study at the end of culture.

During carbon starvation, autolytic cell wall degradation is mainly carried out by extracellular hydrolases (e.g. chitinases, hexose aminidases and glucanases; van Munster et al., 2016). Living hyphae cells produce, melanin (among other things) to protect their cell walls against these hydrolases (Emri et al., 2018). The mutants inhibited in the DUG pathway secreted less chitinase and hexosaminidase into the fermentin, had a lower DCM and produced less melanin than the control strain. In the studies, the deletion strains produced less protease and γGT compared to the control strain.

We also investigated the effect of H_2O_2 treatment on hexose aminidase and extracellular γGT production. H_2O_2 was able to increase the production of both extracellular enzymes in the THM4 mutant tested, but the same treatment had the opposite effect in the control strain.

Since the *ΔdugB* and *ΔdugC* mutants (THM2 and THM3) and *the ΔdugB-ΔdugC* double mutant (THM4) behaved very similarly in the experiments described above, only one mutant (THM4) was selected for further analysis. Although the double deletion of *dugB* and *dugC* increased cellular GSH content and slowed GSH degradation under carbon starvation, the oxidative, salt and congo red (CR) stress tolerance of the THM4 strain was not significantly different from that of the reference strain. However, the THM4 mutant showed increased tolerance to $CdCl_2$.

Double deletion of the *dugB* and *dugC* genes did not significantly alter fungal conidia production on medium containing 10 g l^{-1} glucose. The use of agar plates with a starting glucose concentration of 40 g l^{-1} reduced radial growth and increased conidial production of the control strain. The elevated glucose concentration also reduced radial growth in the THM4 mutant but did not increase conidia production. In addition, the balance between sexual and asexual development was shifted towards sexual development, with the development of cleistothecia (primordium) being initiated and specific conidia production being reduced, and the proportion of colonies covered with conidia was also reduced.

For cultures containing 10 g l^{-1} glucose, the addition of 10 mM GSH or dithiothreitol (DTT) reduced radial growth and conidia formation in both strains, and in the presence of 10 mM GSH, the THM4 mutant produced significantly less conidia than the control strain. The difference between the conidia production of the two strains in the presence of 10 mM GSH was even more striking in the medium containing 40 g l^{-1} glucose.

For our transcriptomic studies, we used RNAseq data from carbon starved submerged cultures of the THM4 double mutant and THS30 control strain, with glucose-grown cultures as reference. The lack of an available carbon source significantly modified the transcriptome of the cultures. Double deletion of the *dugB* and *dugC* genes had only a small effect on the fungal transcriptome, this effect being more pronounced under glucose than under carbon starvation.

The main changes induced by carbon starvation were identified by gene set enrichment (GSE) studies, which revealed that (**i)** fatty acid degradation was up-regulated and nucleotide and amino acid biosynthesis was down-regulated. (**ii**) The autophagy-related genes, extracellular protease, carbohydrate-active enzyme (CAZyme) and genes responsible for cell wall degradation were up- -regulated, and genes for cell wall biosynthesis were down-regulated. (**iii)** Genes for glycolysis, the oxidative pentose phosphate pathway, the citric acid cycle and respiration were down-regulated. (**iv**) Genes for iron-sulfur cluster assembly, ergosterol biosynthesis and genes involved in translation were down-regulated. (**v**) Genes for antioxidant enzyme

(e.g. CatA and CatB catalases, SodA and SodB superoxide dismutases, GlrA glutathione reductase, TrxR thioredoxin reductase and Ccp1 cytochrome *c peroxidase*) were significantly enriched in the group of down-regulated genes. Although antioxidant enzyme genes were not enriched in the group of up-regulated genes, several lesser known antioxidant enzyme genes showed up-regulation: genes for CatC catalase, CpeA catalase-peroxidase, AN7924 peroxidase, AN1131 and AN0785 superoxide dismutases, TrxB thioredoxin reductase and AN5440 cytochrome *c peroxidase.* (**vi**) Genes related to cysteine, methionine and GSH metabolism were also enriched in the down-regulated gene sets of the two strains. *DugA* (putative dipeptidase gene), *ggtA* (γGT gene) and AN5658 (putative γGT gene) were up-regulated, while *gcsA* (putative γ-glutamylcysteine synthetase gene) was down-regulated. The genes for AN12476 GSH synthase and AN2988 putative γ-glutamyl cyclotransferase were also down-regulated in strain THS30 and THM4. In the reference strain, *dugB* was down-regulated and *dugC* was up-regulated. (**vii**) SMGs showed significant enrichment in the up-regulated gene sets: the carbon starvation condition activated the transcription of 206 SMGs in strain THS30 and 209 in strain THM4. Although genes from SMG clusters were generally not enriched in the down-regulated gene sets, genes from some clusters showed significant enrichment. These clusters include the inp cluster and the pkh cluster in the control strain, and the microperfuranone cluster and the "No PKS/NRPS backbone 1" cluster in both strains.

A direct comparison of transcriptome data from carbon starvation cultures (THM4 mutant vs THS30 reference strain) showed that certain SMG clusters (AN7884 cluster, benzaldehyde derivative 1, F9775 hybrid cluster 2, pkg cluster, STC cluster) were more active in the THM4 mutant than in the reference strain. As for the STC cluster, the higher transcriptional activity of this cluster gene in the THM4 mutant was also accompanied by increased STC production. In the *ΔdugB-ΔdugC* double mutant, 24 of the total 40 up-regulated genes compared to the reference strain were members of an SMG cluster.

Direct comparison of transcriptome data from growth cultures (THM4 mutant vs THS30 reference strain) showed that double deletion of *dugB* and *dugC* up-

regulated genes involved in ergosterol biosynthesis, while iron-sulfur cluster assembly genes and the *hacA* gene, which are involved in the uncoiled protein response (UPR), were down-regulated. In addition, down-regulation of several MAPK pathway genes (e.g. *steC*, *sskB*, *pbsA*, *hogA* and *mkkA*), as well as genes involved in developmental processes (conidia formation and ascospore formation; e.g. *flbA*, *flbC*, *flbE*, *nosA*, *rosA*, *nsdC* and *nsdD*) and cell wall biosynthesis was also observed. These transcriptional changes were consistent with the reduced conidial production and altered sexual development of surface cultures observed on glucose-rich medium.

4.2. Investigation of carbon limitation stress response under lactose

We further investigated the physiological changes in three cultures exposed to different carbon stresses (carbon starvation and carbon source limitation in the presence of a disaccharide and a polysaccharide). Washing the mycelia from glucose-containing medium to carbon source-free or lactose/arabinogalactan-containing medium significantly reduced the MTT-reducing activity of the cultures. However, this reduction was only temporary, since after 4 h the MTT-reducing activity of the carbon-stressed cultures started to increase even in the carbon starved cultures. As expected, carbon stress also reduced fungal growth. Compared to the glucose cultures, the greatest biomass reduction was observed in the carbon starved cultures, followed by the arabinogalactan and lactose cultures. Although long-term carbon starvation is usually associated with a DCM decrease, in our case only a small (but not statistically significant) DCM decrease was observed after 12 h.

In all cultures, carbon stress significantly decreased the activity of nitrate reductase and glutathione reductase, while it increased the activity of intracellular β-galactosidase. This increase was greatest in lactose-containing cultures, followed by arabinogalactan-containing cultures and then by carbon-starved cultures. The intracellular SOD activity of the cultures increased on arabinogalactan and under carbon starvation conditions. SOD activity was detectable in the fermentation broth of all four cultures and catalase activity was also detected during carbon starvation.

Carbon stress also increased extracellular protease, γGT, chitinase and β-glucosidase activities, and, in addition, culture on lactose or arabinogalactan, except for carbon starvation, increased extracellular β-galactosidase and cellulase activities.

The presence of the following proteins was detected in the fermentation broth of the carbon-starved cultures: AbnC (AN8007; putative extracellular endo-1,5-α-L-arabinosidase), EglB (AN3418; cellulase); BglA and BglL (AN4102 and AN2828; putative β-glucosidases), ChiB (AN4871; chitinase); EglC (AN7950; putative GPI-anchored glucan endo-1,3-β-D-glucosidase), PepJ (AN7962; protease), CatB (AN9339; catalase) and SodA (AN0241; Cu/Zn-SOD). Correspondingly, protein genes were also up-regulated by the corresponding carbon stress with the following exceptions: We were unable to detect the up-regulation of *eglB* under carbon starvation or the up-regulation of *SodA* and AN8445 in lactose cultures, but we were able to detect the presence of the encoded proteins in the fermented juice. However, for SodA and EglC (detected in carbon starved cultures) and CatB (also detected in carbon starved and lactose starved cultures), the corresponding genes were down-regulated even under carbon stress.

Although sampling for transcriptomic analyses was performed at time points when the utilization of lactose and arabinogalactan had most likely already begun, based on the DCM and MTT reduction patterns. Even so, we observed significant overlap between the genome-wide expression changes of the three cultures

Based on GSE analyses, carbon starvation down-regulated protein synthesis, several elements of primary metabolism (e.g. glucose utilization, amino acid biosynthesis, steroid synthesis), as well as the transcription of several stress genes. On the other hand, however, it up-regulated genes involved in cell wall organization, chitin, xylan and pectin degradation, and fatty acid oxidation. Substitution of glucose with lactose down-regulated several elements of primary metabolism (e.g. glucose utilisation and steroid synthesis), but unlike in carbon starved cultures, no down-regulation of amino acid biosynthesis, ribosome biogenesis and translation was observed. Extracellular polysaccharide utilization, including the metabolism of hexoses and pentoses other than glucose, was up-regulated. Growth on

arabinogalactan down-regulated genes for glucose utilization, amino acid biosynthesis and steroid synthesis. However, as with lactose, down-regulation of high-volume protein synthesis was not observed. Genes involved in extracellular polysaccharide utilization were also up-regulated, but genes involved in pentose or hexose (e.g. galactose, mannose) metabolism were enriched in both the up- and down-regulated gene clusters. The results of GSE analysis of glucose-specific genes suggest that processes related to glucose utilization and growth (e.g. glycolysis, respiration, and biosynthesis of prosthetic groups of steroids, vitamins, cofactors) were up-regulated, whereas polysaccharide catabolic processes and lipid metabolism (e.g. fatty acid oxidation) were down-regulated in glucose-rich cultures compared to other cultures. In parallel, the cultures exposed to carbon stress were characterised by an up-regulation of different elements of carbohydrate catabolism and an down-regulation of some processes, mainly related to growth. Transcription of glycolysis genes was down-regulated by carbon stress, but down-regulation of genes for the oxidative pentose phosphate pathway and the citric acid cycle was only observed in the carbon starved and arabinogalactan cultures, which also showed a growth rate deficit in the experiments.

Carbon stress also significantly affected cell wall homeostasis. In general, decreased expression of genes for synthases, transglycosylases and regulatory proteins and up-regulation of hydrolases was observed, which also coincides with the moderate growth of the cultures. However, for lactose cultures, which showed the most intensive growth among the three stress treatments, the enrichment of the above-mentioned gene clusters was not significant. Among the cell wall hydrolase genes, *engA* (endo-1,3-β-glucanase), *chiB* (endocytinase) and *nagA* (N-acetyl-β-glucosaminidase), which have been shown to be important in the utilization of dead cell walls (autolytic cell wall degradation), were up-regulated in the carbon starved and arabinogalactan cultures (van Munster et al., 2016). Notably, the transcriptional activity of gene clusters responsible for maintaining cell wall integrity was not altered. Melanin production may protect cells from chitinases, including ChiB chitinase (Szilágyi et al., 2013). The *chiB* gene was up-regulated not only in carbon starved and arabinogalactan cultures

but also in lactose-grown cultures. Not surprisingly, the ivo ('ivory') gene cluster responsible for the formation of N-acetyl-6-hydroxytryptophan-type melanin and the gene cluster responsible for the metabolism of aromatic amino acids were also up-regulated in the three treatments, and several cell wall hydrolase genes were down-regulated in the cultures. Autophagy-related genes were identified only in the carbon starved cultures.

The galR and *galX* genes, encoding transcription factors of genes responsible for lactose utilization, were up-regulated only in lactose-containing cultures. This was associated with the up-regulation of genes encoding the oxidoreductive pathway that degrades D-galactose, as well as several known or putative genes encoding lactose permeases and β-galactosidases, including the key β-galactosidase (*lacD*) and lactose permeases (*lacpA, lacpB*) involved in lactose utilization. However, none of the genes of the lactose-degrading Leloir pathway were up-regulated.

Under arabinogalactan, *araR* was up-regulated, but *galR* and *galX* activity was unchanged. Up-regulation of genes in the D-galactose oxidoreductive pathway was also observed, which corresponded to genes that were also activated on lactose, such as *lacD, lacpA, lacpB*, and several other known or suspected lactose permease and β-galactosidase genes. Interestingly, the genes of the Leloir pathway were down-regulated even in the case of carbon starved cultures. The *lacpB* and *lacD*, as well as some other known or putative β-galactosidase genes, were up-regulated even under carbon starvation.

Carbon stress increased the transcription of certain extracellular peptidase (protease) genes in all cultures. The enrichment of up-regulated gene clusters was significant only in carbon starved and arabinogalactan cultures, but even the 10 known/assumed extracellular peptidase genes up-regulated on lactose were associated with detectable protease activity.

All three treatments also significantly upregulated the genes of CAZyme proteins. The up-regulation of AN9166 (putative exo-1,6-galactanase) was also only observed on arabinogalactan. However, many of the up-regulated CAZyme genes may be involved in the utilisation of carbohydrates that were not present in the culture

medium, as β-1,4-endoglucanase/cellulase, β-glucosidase, cellobiohydrolase and cellobiosidase genes were enriched on arabinogalactan, while genes utilising xylosidase and rhamnogalacturonan were also enriched in the up-regulated gene group in all three treatments. Most of the CAZyme genes (65 genes) were observed under arabinogalactan, followed surprisingly by glucose (29 genes), then carbon starved cultures (16 genes) and finally lactose (6 genes). For the arabinogalactan-overexpressed CAZyme genes, several groups of genes belonging to a separate subcategory also showed significant enrichment. However, no such enrichment was observed in the carbon starved strains. Interestingly, only the α-galactosidase gene cluster was up-regulated on lactose, but not the β-galactosidase gene cluster. In fact, on lactose, only *lacD* encoding β-galactosidase, which is essential for lactose utilization, was expressed with significantly higher activity compared to the other cultures. In this respect, the glucose reference cultures were characterized by an enrichment of the β-1,4-endoglucanase/cellulase gene cluster.

Regarding carbon source-specific genes, the transcription factor gene cluster was up-regulated in stress-treated cultures and down-regulated in glucose-specific cultures. In addition to the up-regulation of *galR* and *galX* on lactose and *araR* on arabinogalactan and during carbon starvation, *clrA*, responsible for the regulation of cellulase and xylanase production, *rhaR*, responsible for rhamnose utilization, and *brlA* andresponsible for extracellular peptidase and cell wall hydrolase production, were up-regulated in the carbon stressed cultures. The transcription of the gene for the transcription factor HacA, responsible for unfolded protein response (UPR; endoplasmic reticulum stress), was also increased under carbon stress.

Since the secondary metabolism of cells is largely determined by the quality and quantity of the available carbon source, the transcriptional activity of SMG clusters was also assessed. These gene clusters showed a mixture of up- and down-regulated clusters in all three treatments. Each of the four cultures had a distinctive SMG cluster that showed the highest transcriptional activity in that culture, but the differentially expressed clusters showed an overall similar pattern. Among the most important SMG clusters that were up-regulated by carbon stress, the transcriptional activity of the STC

cluster was prominent, and the formation of the encoded mycotoxin was also detected on TLC plates. Although the down-regulated gene clusters were most abundant on glucose, there were some clusters (microperfuranone cluster, pkh cluster and AN3273 cluster) that showed higher activity in reference cultures compared to the three stressed cultures.

5. Discussion

5.1. GSH metabolism of carbon starved strains

5.1.1 Role of the DUG pathway in the intracellular degradation of glutathione

We have shown that the DUG pathway is involved in the degradation of cytosolic GSH *in A. nidulans*, as the GSH content of mutant cultures decreased more slowly during carbon starvation than that of the reference strain. These data also provided clear evidence that the DUG pathway is not the only GSH-degrading pathway *in A.* nidulans, therefore further studies are needed to clarify the details of GSH degradation. Although the strict negative feed-back control of GSH biosynthesis (Pócsi et al., 2004; Lushchak, 2012) and the alternative GSH-eliminating mechanisms of *A. nidulans* meant that inactivation of the DUG pathway only moderately affected cellular GSH levels, this alone allowed us to investigate the physiological consequences of altered GSH levels.

5.1.2. The connection between redox regulation and stress response to carbon starvation

GSH metabolism may provide a link between the stress response to carbon starvation and redox regulation, as high intracellular GSH concentrations could be utilized by cells as a stored carbon/energy source. Likewise, GSH may also serve as a nitrogen or sulphur reservoir during nitrogen/sulphur limitation or starvation (Pócsi et

al., 2004). Decreased GSH levels may alter the redox state of cells and activate regulatory pathways that control elements of the stress response to carbon starvation.

The studies presented here show that macroautophagy and autolytic cell wall degradation also depend on the redox state of the cells (Emri et al., 2004; Bartoszewska and Kiel, 2011; Deng et al, 2012), as moderate oxidative stress had a beneficial effect on the production of cell wall degrading enzymes, whereas extreme stress induced by H_2O_2 inhibited this process (Emri et al., 2004). Protease and γGT activities were also affected by changes in GSH levels. Since no significant changes were detected in the transcription of genes encoding enzymes responsible for a large part of the extracellular enzyme activity of carbon starved cultures (e.g. ChiB, EngA, PrtA, PepJ and GgtA), it is possible that this segment of redox regulation is exerted at the post-transcriptional level. However, it is also possible that the redox state of cells directly affected production through the regulation of protein secretion.

Although the changes in GSH metabolism altered intracellular ROS levels, the cause of the increased ROS formation is still not fully clear. In addition to the decrease in GSH levels, increased protein secretion (Yu et al., 2016), and a strong change in the production of antioxidant enzymes may have contributed to this. Furthermore, direct ROS production by, for example, NADPH oxidases (Aguirre et al., 2005; Cano-Domínguez et al., 2008) cannot be excluded.

5.1.3. Effect of oxidative stress on the production of secondary metabolites

In our experiments, several SMG clusters were up-regulated by carbon stress (and associated oxidative stress), but some clusters were down-regulated. Surprisingly, inhibition of the DUG pathway further increased the activity of some clusters activated by carbon starvation. A good example of this is the function of the STC cluster: carbon starvation activated the transcription of cluster genes and this up-regulation was significantly greater in the THM4 DUG mutant than in the control strain. Changes in cluster function also altered the amount of STC produced. It has been previously shown that in sexually developing surface cultures, STC production coincides with the

formation of kleistothecia and localizes in the envelope cells (Bayram and Braus, 2012; Ámon et al., 2018). Presumably, its primary function is to protect fruiting bodies from fungivorous arthropods (Staaden et al., 2011; Döll et al., 2013). Given this, it is not surprising that the *dugB-dugC* double deletion, together with the germline processes, also affected STC production.

Consistent with previous observations, moderate oxidative stress can stimulate, whereas strong oxidative stress tends to inhibit, the function of certain SMG clusters (Emri et al., 2015). Unfortunately, this also means that antioxidant treatments aimed at suppressing mycotoxin production may paradoxically even overregulate certain SMG gene clusters by enhancing the formation of undesirable mycotoxins

5.1.4 Signalling aspects of DUG pathway inactivation

The enhanced oxidative, osmotic, cell wall integrity, temperature and antimycotic stress tolerance, which is also shown in literature data for *A. nidulans ΔglrA* strains, clearly indicates the overall importance of GSH in stress tolerance (Sato et al., 2009; Bakti et al., 2017). In contrast, deletion of *dugB* and *dugC* did not increase the fungal tolerance to oxidative stress. It is possible that the negative side effects of GSH accumulation (e.g., excessively reductive internal conditions may prevent proper regulation of the oxidative stress response) may counteract the increased antioxidant capacity. The detrimental consequences of GSH overabundance are well reflected in a previous observation that GSH added to the nutrient medium at high concentrations is specifically toxic to *A. nidulans* (Bakti et al., 2017).

In contrast to oxidative stress tolerance, cadmium tolerance was increased in a well-detectable manner by inactivation of the DUG pathway. This observation is consistent with the hypothesis that the fungus defends itself against this heavy metal by forming a GSH-cadmium complex (Emri et al., 2021). In this case, higher GSH levels are beneficial for the fungus despite their negative side effects. This also implies that the search for cadmium-tolerant mutants may be a better strategy to isolate GSH-overexpressing strains than testing for increased oxidative stress tolerance.

The behaviour of the THM4 mutant differed significantly from that of the wild type strain not only under carbon starvation conditions but also in glucose-grown cultures. There were changes in the transcription of several mitogen-activated protein kinase (MAPK) pathway genes (e.g. *steC, sskB*, *pbsA*, *hogA*, *mkkA*). Since MAPK pathways play a central role in maintaining cellular homeostasis, the consequences of increased GSH levels on these genes may explain why GSH overdose is detrimental to *A. nidulans* (Bakti et al., 2017) and why inactivation of the DUG pathway did not increase oxidative stress tolerance.

In addition to stress responses, MAPK pathways also regulate developmental/differentiation processes in *Aspergillus* species (Duran et al, 2010). Thus, it is not surprising that the transcription of genes involved in the regulation of conidia and ascospore formation (e.g. *flbA*, *flbC*, *flbE*, *nosA*, *rosA*, *nsdC* and *nsdD*) is also altered in strain THM4 under carbon starvation. Consistent with these gene expression changes, we observed reduced conidial formation and impaired regulation of germline processes in the THM4 mutant.

The upstream regulators of the MAPK pathways include the redox-sensing His-Asp phosphorylating (two-component) systems. The *A. nidulans* genome encodes 15 histidine kinases, four response regulators and a histidine-containing phosphotransfer protein. Two of the four response regulators (SskA and SrrA) are involved in the regulation of the oxidative stress response, and SskA acts through the HogA MAPK pathway (Furukawa et al., 2005; Hagiwara et al., 2007). *The Candida albicans* Hog1 MAP kinase enzyme has reactive cysteine side chains, whose redox status changes (in addition to phosphorylation of the enzyme) play an important role in the accumulation of the kinase in the nucleus under nitrosative stress (Herrero-de-Dios et al., 2018). Along with redox-sensitive His-Asp phosphorylation systems, this is another interesting example of how redox changes alter the activity of a MAPK pathway. Overall, we can therefore hypothesize that increased GSH levels caused by inactivation of the DUG pathway may have altered the activity of MAPK cascades through redox-sensing mechanisms, which also altered the transcription of several

genes, including genes encoding proteins of MAPK pathways and genes involved in the regulation of differentiation.

5.2. Carbon limitation stress responses under lactose

5.2.1. Enzymatic "detection" during carbon starvation

The utilization of extracellular polysaccharides is a major challenge for microbes, as they first need to identify the carbohydrates present in the environment in order to secrete the right enzyme or enzyme mix for efficient degradation. This problem is usually solved by selecting "scouting" enzymes (van Munster et al., 2016). These enzymes are "ordinary" CAZyme proteins that cannot fully degrade the polymer by themselves, but can efficiently release some oligomers or monomers energy. The latter compounds (as "regulatory molecules") are recognized by the cells, which activates the increased production of all enzymes required to fully and efficiently degrade the polymer (van Munster et al., 2014).

In our case, none of the tested CAZyme subclasses were enriched in the group of carbon starvation-specific up-regulated genes, and most of them were not even enriched in the group of genes activated for total carbon starvation. These results are concur well with the "secretion of scouting enzymes" strategy, whereby fungal starvation secretes several enzymes at a time to search for alternative carbon sources without upregulate complete gene sets needed for the utilization of a polysaccharide.

During carbon starvation, the utilization of stored compounds (e.g. glycogen or even GSH), autophagy and autolytic cell wall degradation can also provide energy sources for cells (Szilágyi et al., 2013; van Munster et al., 2016). In addition to the exploration of potential carbon sources, intensive extracellular enzyme production is also required for autolytic cell wall degradation. Not surprisingly, genes for ribosome biogenesis and ER-specific processes (such as transport between ER and Golgi vesicles, protein glycosylation and ER stress) are up-regulated early in the carbon stress response (Szilágyi et al., 2013). Although ER-specific processes were not

significantly activated during the late stress response we studied, the expression of *hacA*, which encodes a transcription factor that regulates ER stress, was intense. Thus, it appears that proper ER function is particularly important in the adaptation of cells to carbon stress.

5.2.2 The importance of adaptive prediction in the regulation of CAZyme genes

"Adaptive prediction" is a phenomenon that is used in stress biology to explain "stress cross-protection" (Brown et al., 2019). This means that during a stress response to a given stressor, cells not only upregulate stressor-specific gene sets, but also other elements to potentially prepare for the most likely subsequent stress effects. Consequently, the presence of one type of stressor may increase the tolerance of cells to another type of stressor. Plant cell wall polysaccharides are rarely found in isolation in the natural habitats of fungi. Consequently, the presence of one type of saccharide increases the likelihood that other types are also present. Therefore, it is reasonable to assume that regulatory molecules formed during the degradation of one type of polysaccharide may up-regulate several fungal CAZyme genes that may be required for the recognition or degradation of other polymers that may co-occur ('cross-upregulation').

The arabinogalactan polymer from the larch (*Larix*) wood used in our studies is composed of a β-1,3-D-galactopyranosyl main chain with α-L-arabinofuranosyl (C6'), β-1,6-L-galactobiosyl (C4' or C6') and 4-O-(α-L-arabinofuranosyl)-β-D-galactopyranosyl (C6') units as side chains. Thus, it is not surprising that several genes encoding enzymes potentially involved in the degradation of this polymer, including galactosidase and arabinofuranosidase genes, are up-regulated. Growth on arabinogalactan did not up-regulate autophagy genes, suggesting that cellular energy production shifted towards utilization of arabinogalactan components, but transcription of genes involved in autolytic cell wall degradation (e.g. *chiB, engA, nagA*) remained intense. The gene expression pattern of arabinogalactan cultures also showed high activity of genes involved in the utilization of xylan, galacturonan,

rhamnogalacturonan and cellulose. However, the up-regulation of these genes can no longer be explained by the 'scout enzyme secretion' strategy: several of these genes showed significantly higher transcriptional activity on arabinogalactan than in the other cultures, and in addition, several genes were more active in several subcategories compared to the carbon-starved cultures. This property of arabinogalactan-grown cultures is most likely explained by the cross-upregulaton effect of molecules released during arabinogalactan utilization.

5.2.3. "Tragedy of the commons"

Based on Hardin's publication (1968), in ecological and economic terms, if the strategy for utilizing common goods benefits the individual but not the community, the "tragedy of the commons" occurs, as this process leads to the complete depletion of common resources. The extracellular degradation of polysaccharides results in the release of extracellular mono- and oligosaccharides, which are freely accessible to any microorganism in the degradation environment. In this sense, the process creates microbial "public goods". If some microbes utilize these public goods but do not invest energy in secreting enzyme-degrading enzymes (even though they could; "facultative cheats"), they may gain an advantage over their enzyme-secreting counterparts (Smith and Schuster, 2019). Since it is in the interest of every cell to use as much of the commons as possible with as little enzyme secretion investment as possible, a rapid depletion of the commons can be predicted if only enzyme-secreting individuals cannot prevent the (facultative) cheaters from gaining ground. Several options are possible: e.g. limiting the diffusion of liberated compunds by efficient transport and/or attachment of the extracellular enzyme to the cell surface, or spatial isolation of enzyme-secreting individuals from decoys (Lerch et al, 2022). The extracellular degradation of biopolymers is generally regulated by feed-back inhibition and feed-back repression (Glass et al., 2013; Wang and Lu, 2016). These negative feedback mechanisms, in addition to the rapid utilization of released molecules, may play an

important role in limiting facultative decoys by preventing the rapid accumulation of public goods

5.2.3.1. The regulatory role of lactose in controlling facultative cheaters

Although lactose in its free form is very rarely found in nature (typically only in the milk of mammals), both β-galactosidic bonds (e.g., in xyloglucans, rhamnogalacturonans, and arabinogalactan-proteins) and α-galactosidic bonds (e.g., in galactomannans, galacto-glucomannans, and extensins) are frequently present in plant cell wall polysaccharides (Held et al., 2015). Galactose is also part of the fungal cell wall components galactomannan (galactofuran side chains) and galactosaminogalactan (α-1-4-galactoside and N-acetylgalactosamine side chains). Not surprisingly, the *A. nidulans* genome, like that of many other fungi, contains a number of α- and β-galactosidases and some galactanase genes that serve to hydrolyse these polysaccharides and the oligo- and disaccharides released during their degradation.

The lactose-containing cultures were characterized by active transcription of genes directly involved in lactose utilization, such as *lacD* β-galactosidase, *lacpA* and *lacpB* lactose permeases, and the D-galactose oxidoreductive pathway. In addition to these, only 81 CAZyme genes were up-regulated on lactose, all but one of which were active on arabinogalactan and under carbon starvation. Lactose also resulted in the induction of fewer extracellular peptidase and fungal cell wall hydrolase genes compared to the other two carbon stresses. Notably, in addition to *lacD*, other β-galactosidase genes were also up-regulated, along with an increased induction of α-galactosidase genes. However, the majority of the up-regulated galactosidase genes are basically involved in the utilization of galactose-containing compounds other than lactose. Fewer of the genes involved in the degradation of galactose-containing polymers (xyloglucan and rhamnogalacturonan-degrading genes, as well as galactanase, arabinofuranosidase and endo-arabinosidase genes) were activated on lactose than in the other treatments. Of the up-regulated genes related to the

degradation of non-galactose compounds (β-1,4-endoglucanase, β-glucosidase, cellobiosidase and cellobiose dehydrogenase genes), 11 were on lactose, more than in the carbon-starved cultures (8 genes) and less than in the arabinogalactan cultures (19 genes).

Together, these changes suggest that the high lactose concentration may have mimicked a situation in which the fungus was exposed to galactose-containing polysaccharides in the environment, which were so efficiently degraded that galactose-containing oligomers began to accumulate outside the cell. In this situation, the importance of searching for alternative nutrients and of up-regulating the genes necessary for the degradation of any co-occurring polysaccharides, activating autophagy or, for example, maintaining autolytic cell wall degradation, is diminished. At the same time, the cells must maintain an optimal (not too high) level of "microbial commons" to prevent the proliferation of facultative decoys. Therefore, the rate of creation and utilisation of potential commons must be kept in balance. At the gene expression level, this has resulted in a CAZyme profile on lactose where more CAZyme genes involved in the degradation of the perceived/predicted polysaccharide are up-regulated than during carbon starvation, but fewer than in the presence of arabinogalactan. It is highly likely that the effect of lactose is closely related to its concentration and that the molecule acts as a dual regulator (inducing at low concentrations and repressing at high concentrations).

5.2.3.2. A. nidulans as a facultative cheater

Although free glucose is not as rare a molecule in the environment as lactose, its presence is far from abundant in soil and in most natural habitats of *Aspergillus* species. Most of the available glucose occurs as monomers of various α- and β-glucans. As far as the plant cell wall is concerned, cellulose, mixed-bonded glucan, xyloglucan and glucomannan are the most abundant glucose-containing compounds, while in the fungal cell wall, β-1,3- and α-1,3-glucans have the most significant glucose content (de Groot et al., 2009; Held et al., 2015).

In our case, glucose-rich conditions up-regulated genes for glycolysis, while genes responsible for autophagy and autolytic cell wall degradation were down-regulated compared to the profile of carbon stressed cultures. The extracellular peptidase genes and CAZyme genes also showed low transcriptional activity. Interestingly, some CAZyme genes had the highest transcriptional activity on glucose. These genes encode enzymes involved (or presumably involved) in the degradation of glucose polymers. The characteristics of the glucose cultures (e.g. the numerous up-regulated glucose-specific genes involved in the degradation of β-glucan) are similar to those observed for lactose. It is conceivable that the high glucose concentration also mimics the situation in *A. nidulans* of having a polysaccharide in its environment that is so efficiently degraded that its monomers have accumulated outside the cell. As a result, the cells began to increase their glucose utilisation or inhibit the release of glucose monomers, and down-regulated processes other than glucans to seek alternative nutrients or to degrade polysaccharides, and autophagy and autolytic cell wall degradation were also repressed. However, the low transcriptional activity of genes for extracellular enzymes (e.g. peptidases or plant and fungal cell wall hydrolases) was more striking on glucose compared to lactose, suggesting that facultative cheating is a more preferable tactic on glucose than on lactose. This may be related to the fact that cells are able to utilize glucose much faster than lactose. Preventing the depletion of glucose as a "public good" would require very intensive glucan degradation, and achieving the high enzyme activity required for this would involve a significant energy expenditure, which is not conducive to cooperation. However, this strategy could easily lead to a proliferation of facultative cheating, leading to a "tragedy of the commons". A good tactic for facultative cheaters is to invest in rapid vegetative growth. Growth, as an autocatalytic process, itself consumes large amounts of glucose as a source of energy and carbon, and newly forming cells will also use glucose for further growth. Consequently, rapid growth allows cells and their progeny to use more of the commons than other, slower growing cells. Not surprisingly, in addition to the groups of genes associated with glucose utilization and vegetative growth, a group of antioxidant enzyme genes has been enriched for glucose-

specific up-regulated genes. This is consistent with the fact that aerobic glucose utilization leads to the formation of reactive oxygen species (ROS) and supports the idea that rapid growth based on aerobic metabolism can be dangerous for microbes (Hallsworth, 2018). Furthermore, the largest clusters of down-regulated secondary metabolite gene clusters were also observed in the gene expression profile of glucose cultures. The down-regulated expression of transcription factor genes, irrespective of the fact that some encode negative regulatory elements, in itself suggests that several processes active under carbon stress are repressed on glucose. Overall, therefore, it can be concluded that cells not only sought to enhance glucose utilisation, but also down-regulated a number of 'redundant' biological processes (including the production of several secondary metabolites) that would otherwise reduce their growth rate, and thus their rapid utilisation of 'microbial commons'.

5.2.4. The importance of regulating CAZyme secretion

A. nidulans, as a typical soil-dwelling filamentous fungus, usually grows on decaying plant remains. The regulation of hundreds of CAZymes and other genes required for the efficient degradation of plant biopolymers is a very complex process in which the "regulatory molecules" generated during polymer utilization play a central role (Glass et al., 2013; Znameroski and Glass, 2013; Wang and Lu, 2016). They are involved in the recognition of compounds present (see "enzymatic detection" during carbon starvation), can trigger cross-regulation (see: "adaptive prediction of available biopolymers" based on the presence of arabinogalactan), or even repress genes high concentrations that may be important in limiting facultative cheating (see: lactose utilization) or even lead to switching to a facultative cheating strategy (see: rapid growth on glucose). A detailed study of the molecular and behavioural ecology of this complex regulatory network may in the future help us to better understand the CAZyme secretion of fungi, which may improve their industrial applicability.

The transcriptional regulation of the CAZyme genes is an example of how cells can initially detect stress (change), but do not yet "know" how to adapt to it. Therefore,

they first make an "scouting response" and then, on the basis of the information gathered, take further steps in the right direction. It is reasonable to assume that, in addition to carbon source limitation stress responses, other stress responses may also have "scouting elements" that can guide the response in the right direction. This would explain the ability of the fungi to adapt so successfully to different (even highly manipulated) environmental factors, even with a modified genetic background.

6. Summary

Our experiments focused on the processing and interpretation of transcriptomic data collected by RNA sequencing of carbon-stressed cultures of model filamentous fungus *A. nidulans*. This approach has allowed us to analyse GSH metabolism, secondary metabolite production, and CAZyme protein formation as part of the carbon stress response in a more comprehensive way than the study of a single enzyme or metabolite.

Previously, the function of the putative proteins of the so-called DUG ("deficient in utilization of glutathione") GSH-degrading pathway (DugA, DugB and DugC) in *A. nidulans* was not yet clear. However, understanding GSH metabolism is essential for deciphering the redox regulation of microorganisms and is also justified from a practical point of view, as fungi may encounter conditions (e.g. carbon stress) not only in nature but also in industrial applications that may stimulate the production of undesirable secondary metabolites (mycotoxins), for which antioxidant treatment is considered a promising option to reduce their formation. Therefore, in the first half of our experiments we investigated the functions of the *dugB* and *dugC* genes *in A.* nidulans. Deletion of *dugB*, *dugC*, or both resulted in a moderate increase in GSH content of mycelia growing on glucose, reduced conidial production and impaired sexual development. Transcriptome data revealed that, consistent with observations, some MAPK pathway genes (e.g. *steC*, *sskB* and *hogA*), as well as genes encoding proteins regulating conidia formation and sexual differentiation (e.g. FlbA, NosA, RosA and NsdC) were down-regulated *in the ΔdugB-ΔdugC* mutant. Deletion of *dugB* and/or *dugC* slowed the depletion of GSH pools during carbon starvation, in addition to reducing ROS accumulation, autolytic cell wall degradation and enzyme secretion, but increased STC production. Our transcriptomic analysis suggests that, in contrast to mycotoxin production, fungal enzyme secretion is regulated at the post-transcriptional level. Furthermore, we also found that GSH likely links starvation to redox regulation, as cells utilize GSH as a stored carbon source, and its depletion

results in redox imbalance, potentially activating carbon stress response signaling pathways.

Understanding the coordinated regulation of the hundreds of CAZyme genes present in fungal genomes also has significant practical relevance. Therefore, in the second phase of our experiments, we compared the behavior of four *A. nidulans* cultures grown in the presence of glucose, lactose, or arabinogalactan, as well as under carbon-starvation conditions. We identified carbon stress-specific changes (weak carbon source or its absence *vs*. glucose) and carbon source-specific changes (a single culture *vs*. all other cultures). Since the availability and quality of the carbon source strongly influence cellular secondary metabolism, we also evaluated the transcriptional activity of SMG clusters. Several CAZyme genes showed carbon stress-specific and/or carbon source-specific up-regulation on arabinogalactan (138 and 62 genes), where in addition to galactosidase and arabinan-degrading enzyme genes, transcriptional activity of cellulolytic, pectinolytic, mannan- and xylan-degrading enzyme genes was observed. On lactose, 81 and 6 (galactosidases, xylosidases and rhamnogalacturonases), under carbon starvation 107 and 16 (rhamnogalacturonases) carbon stress-specific and carbon source-specific up-regulated CAZyme genes were found. On glucose, only a few (29 genes) carbon source-specific inductions were detected, which were typically β-1,4-glucanase genes. Each of the four cultures had a distinct SMG cluster with the highest transcriptional activity in that specific culture, but the overall pattern of differentially expressed clusters was similar. In this study, we also evaluated the behavioural ecology of these traits using the models of "secretion of scouting enzymes", "adaptive prediction", "tragedy of the commons" and "facultative cheating". Additionally, we systematized our knowledge regarding CAZyme production, which may lead to the development of new strategies for producing enzymes necessary for the saccharification of plant materials.

7. Novel findings of the dissertation

1. The DUG pathway is involved in the degradation of cytosolic GSH in the filamentous fungus *Aspergillus nidulans*.

2. The DUG pathway is not the only GSH degradation pathway in A. nidulans; during carbon starvation, the decrease in GSH concentration does not completely cease even in strains lacking an inactive DUG pathway.

3. GSH metabolism links the fungal stress response to carbon starvation and redox regulation: GSH can be utilized as a carbon/energy source during carbon starvation, but its decreasing concentration alters the redox balance of the cells.

4. Moderate oxidative stress may stimulate, while strong oxidative stress tends to inhibit the activity of certain secondary metabolite gene clusters. (Antioxidant treatments aimed at reducing mycotoxin production can paradoxically even upregulate certain SMG clusters, thereby increasing the production of undesirable mycotoxins.)

5. The function of the endoplasmic reticulum (ER) is of great importance in the life of carbon-stressed cultures. This raises the possibility of antifungal strategies based on disruption of fungal ER activity.

6. The microbial ecological approach may help better understand the formation of enzymes involved in the degradation of extracellular polysaccharides, which could lead to the discovery of new molecular regulatory elements.

7. The transcriptional patterns of CAZyme genes can be explained by ecological models such as "enzymatic exploration" (during carbon starvation), "adaptive prediction" (on arabinogalactan), as well as "the tragedy of the commons" and "repression of facultative cheaters" (on lactose and glucose).

8. References

Aguirre, J., Ríos-Momberg, M., Hewitt, D., and Hansberg, W. (2005). Reactive Oxygen Species And Development In Microbial Eukaryotes. *Trends In Microbiology* 13, 111–118.

Ámon, J., Keisham, K., Bokor, E., Kelemen, E., Vágvölgyi, C., and Hamari, Z. (2018). Sterigmatocystin Production Is Restricted To Hyphae Located In The Proximity Of Hülle Cells. *Journal Of Basic Microbiology* 58, 590–596.

Bakti, F., Király, A., Orosz, E., Miskei, M., Emri, T., Leiter, É., et al.. (2017). Study On The Glutathione Metabolism Of The Filamentous Fungus Aspergillus Nidulans. *Acta Microbiologica Et Immunologica Hungarica* 64, 255–272.

Barratt, R. W., Johnson, G. B., and Ogata, W. N. (1965). Wild-Type And Mutant Stocks Of Aspergillus Nidulans. *Genetics* 52, 233–246.

Bartoszewska, M., and Kiel, J. A. K. W. (2011) The Role Of Macroautophagy In Development Of Filamentous Fungi. *Antioxidants & Redox Signaling* 14, 2271–2287.

Bayram, Ö., and Braus, G. H. (2012). Coordination Of Secondarymetabolism And Development In Fungi: The Velvet Family Of Regulatory Proteins. *FEMS Microbiology Reviews* 36, 1–24.

Brown, A. J. P., Gow, N. A. R., Warris, A., and Brown, G. D. (2019). Memory In Fungal Pathogens Promotes Immune Evasion, Colonisation, And Infection. *Trends In Microbiology* 27, 219–230.

Cano-Domínguez, N., Álvarez-Delfín, K., Hansberg, W., and Aguirre, J. (2008). NADPH Oxidases NOX-1 And NOX-2 Require The Regulatory Subunit NOR-1 To Control Cell Differentiation And Growth In Neurospora Crassa. *Eukaryotic Cell* 7, 1352–1361.

Chettri, D., Verma, A. K., and Verma, A. K. (2020). Innovations In Cazyme Gene Diversity And Its Modification For Biorefinery Applications. *biotechnology Reports* 28, E00525.

Chomczynski, P. (1993) A Reagent For The Single-Step Simultaneous Isolation Of RNA, DNA And Proteins From Cell And Tissue Samples. 15, 532–534, 536–537.

Contesini, F. J., Frandsen, R. J. N., and Damasio, A. (2021). editorial: Cazymes In Biorefinery: From Genes To Application. *Frontiers In Bioengineering And Biotechnology* 9, 86.

Davies, G. J., Gloster, T. M., and Henrissat, B. (2005) Recent Structural Insights Into The Expanding World Of Carbohydrate-Active Enzymes. *Current Opinion In Structural Biology* 15, 637–645.

De Groot, P. W. J., Brandt, B. W., Horiuchi, H., Ram, A. F. J., De Koster, C. G., and Klis, F. M. (2009). Comprehensive Genomic Analysis Of Cell Wall Genes In Aspergillus Nidulans. *Fungal Genetics And Biology* 46, S72-S81.

Deng, Y., Qu, Z., and Naqvi, N. I. (2012) Role Of Macroautophagy In Nutrient Homeostasis During Fungal Development And Pathogenesis. 1, 449–463.

Döll, K., Chatterjee, S., Scheu, S., Karlovsky, P., and Rohlfs, M. (2013). Fungal Metabolic Plasticity And Sexual Development Mediate Induced Resistance To Arthropod Fungivory. *Proceedings Of The Royal Society B: Biological Sciences* 280, 20131219.

Duran, R., Cary, J. W., and Calvo, A. M. (2010) Role Of The Osmotic Stress Regulatory Pathway In Morphogenesis And Secondary Metabolism In Filamentous Fungi. *Toxins* 2, 367–381.

Emri, T., Gila, B., Antal, K., Fekete, F., Moon, H., Yu, J.-H., et al.. (2021). Atfa-Independent Adaptation To The Toxic Heavy Metal Cadmium In Aspergillus Nidulans. *Microorganisms* 9, 1433.

Emri, T., Molnár, Z., Pusztahelyi, T., and Pócsi, I. (2004) Physiological And Morphological Changes In Autolyzing Aspergillus Nidulans Cultures. *Folia Microbiologia (Praha)* 49, 277–284.

Emri, T., Szarvas, V., Orosz, E., Antal, K., Park, H., Han, K.-H., et al.. (2015). Core Oxidative Stress Response In Aspergillus Nidulans. *BMC Genomics* 16, 478.

Emri, T., Vékony, V., Gila, B., Nagy, F., Forgács, K., and Pócsi, I. (2018). Autolytic Hydrolases Affect Sexual And Asexual Development Of Aspergillus Nidulans. 63, 619–626.

Furukawa, K., Hoshi, Y., Maeda, T., Nakajima, T., and Abe, K. (2005). Aspergillus Nidulans HOG Pathway Is Activated Only By Two-Component Signalling Pathway In Response To Osmotic Stress. *Molecular Microbiology* 56, 1246–1261.

Gila, B. C., Antal, K., Birkó, Z., Keserű, J. S., Pócsi, I., and Emri, T. (2022). Strategies Shaping The Transcription Of Carbohydrate-Active Enzyme Genes In Aspergillus Nidulans. *Journal Of Fungi* 8, 79.

Gila, B. C., Moon, H., Antal, K., Hajdu, M., Kovács, R., Jónás, A. P., et al. (2021). The DUG Pathway Governs Degradation Of Intracellular Glutathione In Aspergillus Nidulans. *Applied And Environmental Microbiology* 87, E01321-20.

Glass, N. L., Schmoll, M., Cate, J. H. D., and Coradetti, S. (2013) Plant Cell Wall Deconstruction By Ascomycete Fungi. *Annual Review of Microbiology* 67, 477–498.

Hagiwara, D., Asano, Y., Marui, J., Furukawa, K., KANAMARU,.,K Kato, M., et al. (2007). The Sska And Srra Response Regulators Are Implicated In Oxidative Stress Responses Of Hyphae And Asexual Spores In The Phosphorelay Signaling Network Of Aspergillus Nidulans. *Bioscience, Biotechnology, And Biochemistry* 71, 1003–1014.

Hallsworth, J. E. (2018). stress-free microbes lack vitality..*fungal biology* 122, 379–385.

Hardin, G. (1968) The Tragedy Of The Commons. *Science* 162, 1243–1248.

Held, M. A., Jiang, N., Basu, D., Showalter, A. M., and Faik, A. (2015). "Plant Cell Wall Polysaccharides: Structure And Biosynthesis", In *Polysaccharides: Bioactivity And Biotechnology*, Eds. K. G. Ramawat and J.-M. Mérillon (Cham: Springer International Publishing), 3-54.

Herrero-De-Dios, C., Day, A. M., Tillmann, A. T., Kastora, S. L., Stead, D., Salgado, P. S., et al.. (2018). Redox Regulation, Rather Than Stress-Induced Phosphorylation, Of A Hog1 Mitogen-Activated Protein Kinase Modulates Its Nitrosative-Stress-Specific Outputs. *Mbio* 9, E02229-17.

Keller, N. P. (2019) Fungal Secondary Metabolism: Regulation, Function And Drug Discovery. *Nature Reviews Microbiology* 17, 167–180.

Koza, N. A., Adedayo, A. A., Babalola, O. O., and Kappo, A. P. (2022). Microorganisms In Plant Growth And Development: Roles In Abiotic Stress Tolerance And Secondary Metabolites Secretion. *Microorganisms* 10, 1528.

Lerch, B. A., Smith, D. A., Koffel, T., Bagby, S. C., and Abbott, K. C. (2022). How Public Can Public Goods Be? Environmental Context Shapes The Evolutionary Ecology Of Partially Private Goods. *plos Computational Biology* 18, E1010666.

Lushchak, V. Glutathione Homeostasis And Functions: Potential Targets For Medical Interventions, *Journal Of Amino Acids* 2012, E736837.

Pfliegler, W. P., Pócsi, I., Győri, Z., and Pusztahelyi, T. (2020) The Aspergilli And Their Mycotoxins: Metabolic Interactions With Plants And The Soil Biota. *Frontiers In Microbiology* 10.

Pócsi, I., Prade, R. A., and Penninckx, M. J. (2004). "Glutathione, Altruistic Metabolite In Fungi", In *Advances In Microbial Physiology*, (Academic Press), 1-76.

Pócsi, I., Pusztahelyi, T., Saw, L., and Emri, T. (2003). Autolysis Of Penicillium Chrysogenum-A Holistic Approach. *Indian Journal Of Biotechnology*. 2, 293–301.

Sakekar, A. A., Gaikwad, S. R., and Punekar, N. S. (2021). protein Expression And Secretion By Filamentous Fungi. *Journal Of Bioscience* 46, 5.

Sato, I., Shimizu, M., Hoshino, T., and Takaya, N. (2009). The Glutathione System Of Aspergillus Nidulans Involves A Fungus-Specific Glutathione S-Transferase*. *Journal Of Biological Chemistry* 284, 8042–8053.

Smith, P., and Schuster, M. (2019). Public Goods And Cheating In Microbes. *Current Biology* 29, R442-R447.

Spitzmüller, Z., Hajdú, M., Pócsi, I., and Emri, T. (2015a) Degradation Of Glutathione In Aspergillus Nidulans. 66, 242–245.

Spitzmüller, Z., Kwon, N.-J., Szilágyi, M., Keserű, J., Tóth, V., Yu, J.-H., et al. (2015b). Γ-Glutamyl Transpeptidase (Ggta) Of Aspergillus Nidulans Is Not Necessary For Bulk Degradation Of Glutathione. *Archives of Microbiology* 197, 285–297.

Staaden, S., Milcu, A., Rohlfs, M., and Scheu, S. (2011). Olfactory Cues Associated With Fungal Grazing Intensity And Secondary Metabolite Pathway Modulate Collembola Foraging Behaviour. *Soil Biology And Biochemistry* 43, 1411–1416.

Szilágyi, M., Anton, F., Pócsi, I., and Emri, T. (2018). Autolytic Enzymes Are Responsible For Increased Melanization Of Carbon Stressed Aspergillus Nidulans Cultures. *Journal Of Basic Microbiology* 58, 440–447.

Szilágyi, M., Miskei, M., Karányi, Z., Lenkey, B., Pócsi, I., and Emri, T. (2013). Transcriptome Changes Initiated By Carbon Starvation In Nidulans. 159, 176–190.

Van Munster, J. M., Burggraaf, A.-M., Pocsi, I., Szilágyi, M., Emri, T., and Ram, A. F. J. (2016). "Post-Genomic Approaches To Dissect Carbon Starvation Responses In Aspergilli", In *Aspergillus And Penicillium In The Post-Genomic Era*, (Caister Academic Press), 89-112.

Van Munster, J. M., Daly, P., Delmas, S., Pullan, S. T., Blythe, M. J., Malla, S., et al. (2014). The Role Of Carbon Starvation In The Induction Of Enzymes That Degrade Plant-Derived Carbohydrates In Aspergillus Niger. *Fungal Genetics And Biology* 72, 34–47.

Wang, M., and Lu, X. (2016) Exploring The Synergy Between Cellobiose Dehydrogenase From Phanerochaete Chrysosporium And Cellulase From Trichoderma Reesei. *Frontiers In Microbiology* 7, 620.

Yu, Q., Zhang, B., Li, J., Zhang, B., Wang, H., and Li, M. (2016). Endoplasmic Reticulum-Derived Reactive Oxygen Species (ROS) Is Involved In Toxicity Of Cell Wall Stress To *Candida Albicans*. *Free Radical Biology And Medicine* 99, 572–583.

Znameroski, E. A., and Glass, N. L. (2013). Using A Model Filamentous Fungus To Unravel Mechanisms Of Lignocellulose Deconstruction. *Biotechnology For Biofuels* 6, 6.

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List of publications related to the dissertation

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J. Fungi. 8 (1), 1-23, 2022.
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2. **Gila, C. B.**, Moon, H., Antal, K., Hajdú, M., Kovács, R., Jónás, A. P., Pusztahelyi, T., Yu, J. H., Pócsi, I., Emri, T.: The DUG Pathway Governs Degradation of Intracellular Glutathione in Aspergillus nidulans.
Appl. Environ. Microbiol. 87 (9), 1-19, 2021.
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List of other publications

3. Emri, T., Antal, K., Varga, K., **Gila, C. B.**, Pócsi, I.: The Oxidative Stress Response Highly Depends on Glucose and Iron Availability in Aspergillus fumigatus.
J. Fungi. 10 (3), 1-18, 2024.
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4. Vig, I., Benkő, Z., **Gila, C. B.**, Palczert, Z., Jakab, Á., Nagy, F., Miskei, M., Lee, M. K., Yu, J. H., Pócsi, I., Emri, T.: Functional characterization of genes encoding cadmium pumping P1B-type ATPases in Aspergillus fumigatus and Aspergillus nidulans.
Microbiol Spectr. 11 (5), 1-14, 2023.
DOI: http://dx.doi.org/10.1128/spectrum.00283-23
IF: 3.7

5. Emri, T., Sümegi-Győri, V. M., Páll, K., **Gila, C. B.**, Pócsi, I.: Effect of the combinatorial iron-chelation and oxidative stress on the growth of Aspergillus species.
Res. Microbiol. 173 (8), 1-3, 2022.
DOI: http://dx.doi.org/10.1016/j.resmic.2022.103969
IF: 2.6

6. Rácz, D., **Gila, C. B.**, Szőke, L., Széles, A.: N-Stabilizer and Foliar Fertilizer Treatments Enhance Tolerance to Specific Pathogens in Maize (Zea mays L.).
Agric. conspec. sci. 87 (1), 25-33, 2022.

7. Emri, T., Antal, K., **Gila, C. B.**, Jónás, A. P., Pócsi, I.: Stress Responses Elicited by Glucose Withdrawal in Aspergillus fumigatus.
J. Fungi. 8 (11), 1-19, 2022.
DOI: http://dx.doi.org/10.3390/jof8111226
IF: 4.7

8. Emri, T., **Gila, C. B.**, Antal, K., Fekete, F., Moon, H., Yu, J. H., Pócsi, I.: AtfA-Independent Adaptation to the Toxic Heavy Metal Cadmium in Aspergillus nidulans.
Microorganisms. 9 (7), 1-23, 2021.
DOI: http://dx.doi.org/10.3390/microorganisms9071433
IF: 4.926

9. Rácz, D., **Gila, C. B.**, Horváth, É., Illés, Á., Széles, A.: The efficiency of nitrogen stabilizer at different soil temperatures on the physiological development and productivity of maize (Zea mays L.).
Agron. Res. 19 (4), 1888-1900, 2021.
DOI: https://doi.org/10.15159/AR.21.146

10. Antal, K., **Gila, C. B.**, Pócsi, I., Emri, T.: General stress response or adaptation to rapid growth in Aspergillus nidulans?
Fungal Biology. 124 (5), 376-386, 2020.
DOI: http://dx.doi.org/10.1016/j.funbio.2019.10.009
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11. Emri, T., Vékony, V., **Gila, C. B.**, Nagy, F., Forgács, K., Pócsi, I.: Autolytic hydrolases affect sexual and asexual development of Aspergillus nidulans.
Folia Microbiol. 63 (5), 619-626, 2018.
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