This project was planned to be implemented within the frame of an intense collaboration with our partners at the University of Wisconsin – Madison, Wisconsin, USA. Unfortunately, this project, like many others at the same time, was significantly disrupted by the unforeseen COVID-19 pandemic, which significantly hindered the progress of the laboratory-based research work and made planned personal visits impossible. Despite this, we were able to fulfill a significant part of our research obligations, but certain partial tasks of the project still await completion.

Tasks completed:

1. To analyze the function of AtfA and AtfB bZIP-type transcription factors, we constructed the *atfA* and *atfB* gene deletion (*Δ*) and overexpression (OE) strains in all combination (*ΔatfA*, *ΔatfB*, *ΔatfAΔatfB*, *ΔatfAatfB*OE, *ΔatfBatfA*OE, *atfA*OE, *atfB*OE and *atfA*OE*atfB*OE) in *Aspergillus nidulans* (Kocsis et al., 2022a). We tested the stress tolerance of each mutant in the presence of osmotic, heavy metal and cell wall stress generating agents. Overexpression of *atfB* either alone or together with *atfA* as well as the deletion of *atfA* resulted in heavy metal stress sensitive phenotypes while the *ΔatfB* mutant was moderately tolerant to $CdCl_2$. In addition, the *ΔatfB* mutant showed reduced growth on solid medium containing 1.5 M NaCl meanwhile this mutant was the most tolerant to 2 M sorbitol. The *atfA*OE*atfB*OE double overexpression mutant showed an increased tolerance to NaCl. Only the *ΔatfA* mutant displayed moderate tolerance to the cell wall stress inducing agent, Congo Red. Heat stress (50 $^{o}$C, 10 min) reduced the viability of *ΔatfB* mutant and increased the viability of the *ΔatfAatfB*OE and *ΔatfBatfA*OE mutants. Deletion of *atfA* significantly decreased the number of conidiospores both in the *ΔatfA* as well as in the *ΔatfAΔatfB* mutants. Reduced conidiospore formation was observed in the *atfB*OE strain, while increased levels of *abaA* (element of the central regulatory pathway of conidiogenesis) mRNA and size of conidiospores were detected. Interestingly, the overexpression of *atfA* in the *ΔatfB* mutant increased the number of asexual spores of *A. nidulans*. Sexual fruiting body (cleistothecium) formation was diminished in the *ΔatfA* and the *ΔatfAΔatfB* mutants, while significantly elevated in the *ΔatfB* and the *ΔatfBatfA*OE strains. Unexpectedly, the mycotoxin sterigmatocystin (ST) was decreased to undetectable levels in the *ΔatfA* mutant, yet ST production was restored in the *ΔatfAΔatfB* mutant. Levels of ST were also relevantly decreased in the *ΔatfAatfB*OE, *ΔatfBatfA*OE and *atfA*OE*atfB*OE mutants (Kocsis et al., 2022a). These latter findings coincide with defected/considerably decreased mycotoxin productions most recently reported for the *Fusarium verticillioides ΔFvatfA* (Szabó et al., 2020) and *Aspergillus flavus ΔAflatfA* (Wang et al., 2022; Zhao et al., 2022), which observation may pave the way for, for example, the development of novel, RNA interference-based, host-induced mycotoxin control technologies.

2. We also performed genome-wide expression studies with Illumina RNAseq high-throughput sequencing technique to understand the regulatory roles of the bZIP transcription factors AtfA and AtfB in both vegetative tissues and conidia harvested from unstressed and oxidative stress (menadione sodium bisulphite, MSB) exposed *A. nidulans* surface agar cultures (Kocsis et al., 2022b). Comparative transcriptomic analyses were carried out using the control, *ΔatfA*, *ΔatfB*, *ΔatfAΔatfB* mutant strains.

The main findings are:

Both MSB treatment and the deletion of *atfA* downregulated the expression of *atfB*. Based on the latter observation, AtfA can regulate the AtfB-dependent genes *via* activating *atfB* expression. In

untreated mycelia, we have found a higher number of differentially expressed genes (DEGs) in the *ΔatfA* strain than in the *ΔatfB* mutant.

Compared to untreated cultures, MSB treated mycelia showed a lower number of DEGs in the *ΔatfA* or *ΔatfB* mutants *vs*. the control strain. Merely 9 AtfB-dependent genes were found in MSB treated mycelia and most of them were also AtfA-dependent. The observed differences between DEGs of the MSB treated and untreated cultures suggested that AtfA regulated distinct genes under different culture conditions.

More DEGs were identified in conidia harvested from either *ΔatfA* or *ΔatfB* cultures, supporting the view that both AtfA and AtfB play more global regulatory roles in conidiospores than in vegetative mycelia. Interestingly, MSB treatment of conidia did not decrease *atfB* mRNA levels, and most of the *atfB* deletion DEGs were also found in *ΔatfA* cultutres. In general, DEGs in conidia showed low overlap with DEGs found in the corresponding mycelial samples.

Functional category analyses of DEGs have revealed that AtfA regulated some glycolytic and iron-sulfur cluster assembly genes. Moreover, phosphorelay response regulator genes were enriched in all AtfA-dependent gene sets except the untreated mycelial samples. Genes coding for catalase and histidine-containing phosphotransfer protein were also regulated by AtfA under all tested experimental conditions.

Surprisingly, only 23 genes were solely dependent on AtfB considering all transcriptomics data sets, including a putative α-glucosidase (*agdB*), a putative α-amylase, *calA* involved in early conidial germination and an alternative oxidase. Therefore, AtfA and AtfB seem to occupy asymmetric positions in the regulatory network with the dominance of AtfA (Kocsis et al., 2022b).

3. To determine the consensus DNA binding sequence for AtfA, we have already constructed a 3XFLAG-tag labeled AtfA carrying strain for ChIP DNA seq analysis. In this *ΔatfA* strain, the expression of *atfA* is under the control of the native *atfA* promoter. Currently, we are optimizing ChIP DNA seq experiments to identify genes with AtfA binding site(s) in their promoter regions. The optimization involves DNA crosslinking (paraformaldehyde or formaldehyde), physical shearing of DNA-protein complex with sonication (duration, repeats of sonication) and also the anti-FLAG antibody dilution.

4. To confirm the feasible AtfA-AtfB heterodimer formation *in vivo*, we constructed strains to perform bimolecular fluorescence complementation experiments (BiFC). AtfA and AtfB were fused with the N-terminal part and the C-terminal part of YFP, respectively, and both constructs were co-expressed under the control of the *alcA* promoter. Possible interactions between AtfA and AtfB were visualized by confocal fluorescence microscopy in both vegetative tissues and conidia in oxidative stress (MSB, *tert*-butyl hydroperoxide and diamide) exposed and not exposed cultures. Unfortunately, we could not confirm any physical interactions between AtfA and AtfB under the tested experimental conditions. In parallel, we also expressed both AtfA and AtfB proteins in *Escherichia coli* for FRET analysis to demonstrate any possible AtfA-AtfB interactions *in vitro*. Both bZIPs were expressed in *E. coli* ORIGAMI strain transformed with the pET32 plasmids carrying His-tag labelled proteins by IPTG induction. Proteins were purified

with Ni agarose affinity chromatography. The native form of the purified proteins finally processed by enterokinase cleavage. Protein labeling for FRET analysis is now in progress.

5. To complement our AtfA-AtfB interaction studies, we also published a paper on the possible interactions between two other important bZIP-type transcription factors, NapA and RsmA (Bákány et al., 2021). We confirmed that NapA was crucial in the oxidative stress defense of the fungus, and we have also demonstrated that NapA regulated sterigmatocystin (ST) production *via* modulating intracellular reactive species levels meanwhile RsmA controlled the biosynthesis of ST independently of the redox regulation of the cells.

We further studied the effect of gene manipulations, including both gene deletion and overexpression, of *napA* and *rsmA*, on superoxide production, mitochondrial morphology and hyphal diameter of *A. nidulans* (Bákány et al., 2022). We found that reactive oxygen species production was influenced by both gene deletion and overexpression of *napA* under *tert*-butyl hydroperoxide (*t*BOOH) induced oxidative stress. Expression of *napA* negatively correlated with mitochondrial volumetric ratio as well as ST production of *A. nidulans*. High *rsmA* expression level was accompanied with increased relative superoxide ratio in the second hyphal compartment. A negative correlation between the expression of *rsmA* and catalase production or mitochondrial volumetric ratio was also confirmed by statistical analysis. Hyphal diameter was irrespective of either *rsmA* and *napA* expression as well as *t*BOOH (c=0.2 mM) treatment (Bákány et al., 2022).

6. We wrote an invited (by Applied Microbiology and Biotechnology) comprehensive review focusing on the impact of bZIP-type Atf1/AtfA/FvAtfA-orthologous global regulators in fungi. We demonstrated that these transcription factors play an important role in the orchestration of vegetative growth, sexual and asexual development, stress response, secondary metabolite production as well as virulence in both human and plant pathogens (Leiter et al. 2021).

7. We re-evaluated our large-scale DNA chip-based transcriptomics data previously gained by us in stress-exposed and unstressed *A. nidulans* control and *ΔatfA* cultures (Antal et al., 2020). We managed to identify key elements of the MSB stress response when MSB treatments were compared to all other tested culture conditions including unstressed and stressed (altogether 9 different stress treatments) cultures. Furthermore, the versatile transcriptomic effects of *atfA* gene deletion was related to the reduced transcription of several phosphorelay signal transduction system genes, including *fphA*, *nikA*, *phkA*, *srrB*, *srrC*, *sskA* and *tcsB* (Antal et al., 2020). The deletion of *atfA* enhanced the expression of several secondary metabolism cluster genes independently of the stress-type (Antal et al., 2020).

8. We also evaluated the role of the AtfA transcription factor in the $Cd^{2+}$ stress tolerance of *A. nidulans* (Emri et al., 2021). Unexpectedly, the deletion of atfA did not change the $Cd^{2+}$ tolerance of the fungus, and the expression patterns of genes encoding cadmium pumps and those responsible for elevated intracellular Cys levels were not altered significantly in the *ΔatfA* mutant. Nevertheless, genome-wide cadmium stress responses observed in the *ΔatfA* and control strains indicated that the cells lacking AtfA could compensate efficiently for the loss of this transcriptional regulator. These observations highly supported the view that fungal cells have

highly flexible regulatory mechanisms to cope with environmental stress. In this study, the expression of "Phospho-relay response regulator" genes were under AtfA control (Emri et al., 2021).

9. A selection of our latest fungal stress biological studies was presented at The Third International Symposium on Fungal Stress – ISFUS, held in São José dos Campos, São Paulo, Brazil in 2019 (Alder-Rangel et al., 2020).

10. Some other, recently published papers from our laboratory help our current AtfA-AtfB bZIP research only in an indirect way. Nevertheless, on-going and future fungal stress biological studies by our team will also aim at the elucidation of the regulatory functions of AtfA (and AtfB) in carbon starvation (Emri et al., 2022a; Gila et al., 2022) and various combinatorial stress responses (Emri et al., 2022b). To shed light on the possible AtfA-controlled elements of the glutathione metabolism of the Aspergilli (Gila et al., 2021) is another challenge for the forthcoming years. We are constructing further *atfA* gene deletion mutants in other *Aspergillus* spp. as well and, hopefully, will have the chance to extend our studies not only for various further environmental stress types but for other industrially and/or biomedically important *Aspergillus* species too. We can plan and relatively easily implement these studies because some of these experimental conditions have been thoroughly optimized in the publications cited here. Interestingly, some of the most frequently used stressors, like Congo Red, a stain causing cell wall integrity stress, may change their chemical structure and behavior depending on the culture conditions (Csillag et al., 2022) – a factor, which is largely overlooked even in highly sophisticated transcriptomics studies.

Own publications cited in this report:

Alder-Rangel A, Idnurm A, Brand AC, Brown AJP, Gorbushina A, Kelliher CM, Campos CB, Levin DE, Bell-Pedersen D, Dadachova E, Bauer FF, Gadd GM, Braus GH, Braga GUL, Brancini GTP, Walker GM, Druzhinina I, Pócsi I, Dijksterhuis J, Aguirre J, Hallsworth JE, Schumacher J, Wong KH, Selbmann L, Corrochano LM, Kupiec M, Momany M, Molin M, Requena N, Yarden O, Cordero RJB, Fischer R, Pascon RC, Mancinelli RL, Emri T, Basso TO, Rangel DEN. (2020) The Third International Symposium on Fungal Stress – ISFUS. Fungal Biol. 124(5):235-252. doi: 10.1016/j.funbio.2020.02.007. **Q1**

Antal K, Gila BC, Pócsi I, Emri T. (2020) General stress response or adaptation to rapid growth in *Aspergillus nidulans*? Fungal Biol. 124(5):376-386. doi: 10.1016/j.funbio.2019.10.009. **Q1**

Bákány B, Yin WB, Dienes B, Nagy T, Leiter É, Emri T, Keller NP, Pócsi I. (2021) Study on the bZIP-type transcription factors NapA and RsmA in the regulation of intracellular reactive species levels and sterigmatocystin production of *Aspergillus nidulans*. Int J Mol Sci. 22(21):11577. doi: 10.3390/ijms222111577. **D1**

Bákány B, Antal R, Szentesi P, Emri T, Leiter É, Csernoch L, Keller NP, Pócsi I, Dienes B. (2022) The bZIP-type transcription factors NapA and RsmA modulate the volumetric ratio and the relative superoxide ratio of mitochondria in *Aspergillus nidulans*. Lett Appl Microbiol., submitted, under review

Csillag K, Emri T, Rangel DEN, Pócsi I. (2022) pH-dependent effect of Congo Red on the growth of *Aspergillus nidulans* and *Aspergillus niger*. Fung Biol., in press, doi: 10.1016/j.funbio.2022.05.006. **Q1**

Emri T, Gila B, Antal K, Fekete F, Moon H, Yu JH, Pócsi I. (2021) AtfA-independent adaptation to the toxic heavy metal cadmium in *Aspergillus nidulans*. Microorganisms. 9(7):1433. doi: 10.3390/microorganisms9071433. **Q2**

Emri T, Antal K, Gila B, Jónás AP, Pócsi I. (2022a) Stress responses elicited by glucose withdrawal in *Aspergillus fumigatus*. J Fungi (Basel). 8(11):1226. doi: 10.3390/jof8111226. **Q1**

Emri T, Sümegi-Győri VM, Páll K, Gila BC, Pócsi I. (2022b) Effect of the combinatorial iron-chelation and oxidative stress on the growth of *Aspergillus* species. Res Microbiol. 173(8):103969. doi: 10.1016/j.resmic.2022.103969. **Q2**

Gila BC, Moon H, Antal K, Hajdu M, Kovács R, Jónás AP, Pusztahelyi T, Yu JH, Pócsi I, Emri T. (2021) The DUG pathway governs degradation of intracellular glutathione in *Aspergillus nidulans*. Appl Environ Microbiol. 87(9):e01321-20. doi: 10.1128/AEM.01321-20. **D1**

Gila BC, Antal K, Birkó Z, Keserű JS, Pócsi I, Emri T. (2022) Strategies shaping the transcription of carbohydrate-active enzyme genes in *Aspergillus nidulans*. J Fungi (Basel). 8(1):79. doi: 10.3390/jof8010079. **Q1**

Kocsis B, Lee MK, Yu JH, Nagy T, Daróczi L, Batta G, Pócsi I, Leiter É. (2022a) Functional analysis of the bZIP-type transcription factors AtfA and AtfB in *Aspergillus nidulans*. Front Microbiol. 13:1003709. doi: 10.3389/fmicb.2022.1003709. **Q1**

Kocsis B, Lee MK, Antal K, Yu JH, Pócsi I, Leiter É, Emri T. (2022b) Genome-wide gene expression analyses of the AtfA/AtfB-mediated menadione stress response in *Aspergillus nidulans*. Cells, submitted, under review

Leiter É, Emri T, Pákozdi K, Hornok L, Pócsi I. (2021) The impact of bZIP Atf1ortholog global regulators in fungi. Appl Microbiol Biotechnol. 105(14-15):5769-5783. doi: 10.1007/s00253-021-11431-7. **Q1**

Other publications cited in this report:

Szabó Z, Pákozdi K, Murvai K, Pusztahelyi T, Kecskeméti Á, Gáspár A, Logrieco AF, Emri T, Ádám A., Leiter É, Hornok L, Pócsi I. (2020) *FvatfA* regulates growth, stress tolerance as well as mycotoxin and pigment productions in *Fusarium verticillioides*. Appl Microbiol Biotechnol. 104:7879-7899. doi: 10.1007/s00253-020-10717-6.

Wang X, Zha W, Yao B, Lan L, Wang S. (2022) Genetic interaction of global regulators AflatfA and AflatfB mediating development, stress response and aflatoxins B1 production in *Aspergillus flavus*. Toxins 14(12):857. doi: 10.3390/toxins14120857.

Zhao Q, Pei H, Zhou X, Zhao K, Yu M, Han G, Fan J, Tao F. (2022) Systematic characterization of bZIP transcription factors required for development and aflatoxin generation by high-throughput gene knockout in *Aspergillus flavus*. J Fungi (Basel). 8(4):356. doi: 10.3390/jof8040356.