INTRODUCTION

Beside the linker histones, the B-type high mobility group box domain proteins (HMGB), as architectural components of the chromatin, participate in various functions of chromatin, such as recombination, repair, inducing or repressing gene expressions. They are able to interact with both DNA and protein components of the chromatin through their HMG-box domains. Amongst the fungal HMGB proteins, role of the yeast HMGB proteins was studied in detail, providing vast knowledge on the mode-of-action, molecular- and physiological role of HMGB proteins. However, very few data is known about the role of HMGB proteins of filamentous fungi. Detailed study on the function of mitochondrial HMGB protein of *P. anserina* (mtHMG1) and *A. nidulans* (HmbB) revealed their role in the maintenance of mitochondrial genome and additionally, nuclear roles were also proposed. Besides the mitochondrial HMGB proteins, four nuclear HMGB proteins were identified in *P. anserina* and two in *A. nidulans* (HmbA and HmbC). A recent study on the HMGB proteins of the heterothallic fungus, *P. anserina*, revealed that they are involved in the sexual development and fruiting body formation of the fungus. The homothallic *A. nidulans* undergoes sexual development upon certain environmental conditions via the coordinated functioning of various regulators and transcription factors. Amongst them, VeA plays a key role in the light-dependent coordination of asexual- and sexual development and couples the sexual development with the production of the carcinogenic secondary metabolite, sterigmatocystin (STC). In this study we aimed to elucidate the role of *A. nidulans* HMGB proteins in the sexual development and find out whether the HMGB functions are dependent on VeA.

A. nidulans produces a wide range of secondary metabolites, including STC. STC has toxic and carcinogenic effects similar to B1 type aflatoxins. According to the literature, sexual differentiation and STC

production are coupled biological processes in *A. nidulans*. It is quite possible that coupling these biological processes endows the fungus with the ability to defend its sexual reproductive structures from fungivore insects and compete for environmental niches. Despite that the two processes are coupled, the intracolonial site of STC production had never been studied, yet.

AIMS

We aimed to study the role of *A. nidulans* HMGB proteins HmbA, HmbB and HmbC in the sexual development by the analysis of *hmbAΔ*, *hmbBΔ* and *hmbCΔ* mutants and monitor the changes in transcript levels of sexual development related transcription factors in the mutants. Furthermore, we aimed to assess possible functional interaction between VeA and the HGB proteins by comparing the phenotype of *hmb* deletions in veA^{+} and *veA1* genetic background. To achieve our goals we planned to

- obtain *hmbA*, *hmbB* and *hmbC* deletions in veA^{+} genetic background by genetic crosses
- characterize sexual structures of *hmbAΔ*, *hmbBΔ* and *hmbCΔ* mutants in comparison to $hmbA^{+}$ $hmbB^{+}$ $hmbC^{+}$ control both in veA^{+} and in *veA1* genetic background
- carry out transcript analysis of transcription factors involved in sexual development in veA^{+} *hmbAΔ*, *hmbBΔ* and *hmbCΔ* mutants in comparison to veA^{+} $hmbA^{+}$, $hmbB^{+}$ and $hmbC^{+}$ control strains.

In order to study the intracolonial site of STC production we aimed to:

- reveal the role of *stcO* gene in STC biosynthesis by detection of STC in control and *stcOΔ* strains by TLC
- visualize the intracolonial site of STC production *in vivo* through the development and application of a reporter system using the

promoter of one of the putative STC biosynthetic gene, *stcO*, fused with the gene of the cyan fluorescent protein (*ycfp*).

APPLIED METHODS

Cultivation of cells used in experiments

Maintenance of *A. nidulans* strains, cultivation on solid and in liquid medium.

Molecular techniques:

DNA and RNA isolation from filamentous fungus, cDNA synthesis, *protoplasts* isolation and transformation of *A. nidulans*, PCR, qPCR, RT-qPCR, Double-Joint PCR, DNA gel electrophoresis.

Other methods

Southern-hybridization, homo- and heterothallic crossing of *A. nidulans*, fluorescence microscopy, thin layer chromatography (TLC)

RESULTS

Role of HmbA, HmbB and HmbC in the sexual development of *A. nidulans*

Through the study of sexual development of control and *hmbA*, *hmbB* and *hmbC* deletion mutants both in *veA*$^{+}$ and *veA1* genetic background we revealed that HmbA, HmbB and HmbC architectural chromatin components of *A. nidulans* are required for normal sexual development, especially for the formation and viability of ascospores and the spatial distribution of cleistothecia. By studying *hmbA*, *hmbB* and *hmbC* deletions in both *veA*$^{+}$ and *veA1* background, we investigated the possible functional interaction of these HMGB proteins with VeA, the master regulator of sexual development.

The *hmbAΔ*, *hmbBΔ* and *hmbCΔ* strains show defect in ascospore production and viability to various extent. The *hmbA* deletion resulted in nearly sterile cleistothecia with less than 10 ascospores inside, which were remarkably able to germinate with an estimated 30% rate. The *hmbB* deletion resulted in a drastic decrease both in the numbers and the viability of ascospores, whereas a drastic decrease was mainly observed in the ascospore viability but not in the number of ascospores in the case of the *hmbC* deletion. Reddish granular amorphous (RGA) material was detected in all three deleted mutants; amongst them the *hmbCΔ* strain accumulated it to a greater extent which was more pronounced in the *veA1* background. The *veA*$^{+}$ *hmbCΔ* cleistothecia contained an equal number of immature asci and mature ascospores, however the *veA1 hmbCΔ* cleistothecia were either barren or fertile. The fertile *veA1 hmbCΔ* cleistothecia mostly contained free ascospores and an increased amount of RGA material. Furthermore, the abundance of the *veA1 hmbCΔ* cleistothecia was reduced in the *veA1* background. Both the *hmbA* and

hmbB deletions caused a delay in the time-course of the sexual development independently of VeA. However, the delay in the time-course of sexual development observed in the *veA*$^{+}$ *hmbCΔ* strain in comparison to that of the *veA*$^{+}$ control was more pronounced in the *veA1* background. Considering the above described *hmbCΔ* phenotypes, we propose that HmbC functionally interacts with VeA.

HmbA and HmbB might be involved in sensing and responding to the change of environmental oxygen-level. The distribution of the cleistothecia was dependent on oxygen-exposure in the *hmbAΔ* mutants, since only the strict exclusion of oxygen resulted in a wild type-like distribution of cleistothecia. The normal-sized cleistothecia formation in the *hmbBΔ* strain required a medium level of oxygen-exclusion.

The transcript analysis of regulatory genes involved in sexual development revealed that HmbA, HmbB and HmbC seem to be equally important for the normal expression of *matB* (codes for MAT1-1) and *matA* (codes for MAT1-2) genes at four days after the initiation of sexual development, however neither of the *hmbB* and *hmbC* deletion phenocopied fully the *matBΔ* or *matAΔ* phenotypes. Deletion of MAT1-1 coding *matB* and MAT1-2 coding *matA* results in Hülle cell formation and cleistothecium production, however the cleistothecia are free of ascospores and contain only RGA materials. The MAT gene deletion phenotype was frequently, but not always observed in the *veA1 hmbCΔ* strain, which indicates that HmbC functionally interacts with VeA. On the other hand, the random occurrence of barren cleistothecia amongst fertile cleistothecia might reflect a sporadic compensation for the loss of *hmbC* in the *veA1* background. We cannot exclude the possibility that HmbA, HmbB and HmbC directly influence the gene expression of MAT-regulated genes, and that they can provide the functional backups for each other's loss. Such a compensatory effect had already been reported in the case of other types of linker proteins. Mice contain eight subtypes of the

linker H1 histone that are differentially expressed during development and these H1 histone variants can compensate for each other's loss in homozygous knockout mouse models.

Only the *hmbAΔ* phenotype shares all the characteristics of the *matBΔ* and *matAΔ* phenotypes. The deletion phenotypes of *hmbBΔ* and *hmbCΔ* resemble to that of the *matBΔ* or *matAΔ* in terms of RGA material accumulation inside the cleistothecia. It is reasonable to think that besides the pronounced downregulation of MAT genes, the qualitatively different perturbation in the expression of the sexual-differentiation-involved genes further contributes to the *hmbAΔ*-, *hmbBΔ*- and *hmbCΔ*-specific phenotypes. For example, the observed downregulation of *medA*, *stuA*, *atfA* and *hhoA* might contribute to the *hmbAΔ* and *hmbCΔ* phenotypes. MedA and StuA transcription factors are important in cleistothecium formation, but we observed that the downregulation of these genes in the mutants did not inhibit the production of cleistothecia. Therefore *medA* and *stuA* downregulations should not be accounted for the mutant phenotypes. AtfA is known as a repressor of sexual development, however this transcription factor is implicated in oxidative- and osmotic stress-defence and contributes to the viability of conidiospores. The *atfA* deletion results in the drastic decrease of conidiospore viability, which worsens upon prolonged storage-time. Although the viability of ascospores in the *atfA* mutant was not investigated, a pivotal role of AtfA in the viability of ascospores seems to be reasonable to suppose. We included the study of H1 histone in our experiments, as we know that the *hhoA* deletion mutant behave as wild type in various aspects, such as during asexual and sexual reproduction; C- and N-source utilization; performance against various environmental stresses; and nucleosome positioning. Here we uncovered that the expression of *hhoA* is governed by HmbA and HmbC, which might provide the starting point of further efforts to reveal the exact role of H1 histone.

Genes *grrA* and *cpcA* were downregulated only in *hmbAΔ*, therefore they might explain the *hmbAΔ* phenotype. The phenotype of the *grrAΔ* mutant shows resemblance with that of *hmbAΔ* in terms of the lack of ascospore production, however empty asci and ascogenous hyphae are found in *grrAΔ*. Downregulation of *grrA*, together with the downregulation of mating-type coding MAT genes, might be crucial explanatory factors for the *hmbAΔ* phenotype. The transcription factor CpcA arrests cleistothecium maturation upon amino acid starvation; in the absence of CpcA mature cleistothecia are formed. Therefore downregulation of *cpcA* should not be accounted for the *hmbAΔ*-specific phenotype. The *velB, flbC, lreA* and *lreB* genes are downregulated only in the *hmbCΔ* strain. Deletion of *flbC* results in abundant cleistothecium production, therefore downregulation of *flbC* might contribute to the wild type-like abundance of cleistothecia in the veA^{+} *hmbCΔ* strain. The *hmbC* deletion in the *veA1* background resulted in a scarce cleistothecium production. The colonies frequently contained sectors, which were poor in conidia but rich in cleistothecia. This phenotype might be explained with a supposed intracolonial fluctuation in the expression level of *flbC*, however, future studies should test this hypothesis. Downregulation of *lreA* and *lreB* in *hmbCΔ* might be the clue for the extreme delay in the time-course of sexual development in *veA1* background. LreA, LreB, FhpA and VeA form the 'light regulator complex' in the nucleus. Since the loss of the nuclear localization signal in VeA protein (creates the truncated VeA1 protein) results in a decreased presence of VeA1 in the nucleus, depletion of LreA and LreB components of the 'light regulator complex' by the lack of HmbC might result in a delay in the progress of sexual development. Downregulation of *velB* in the *hmbCΔ* strain might contribute to the decreased viability of ascospores through compromising the functioning of the VosA-VelB complex that is important for the maturation of the ascospores. The upregulation of *nsdC*, AN3667 and *fhp*A might contribute to the success of cleistothecium production in the

mutants, by being positive regulators of sexual development. The positive regulatory role of AN3667 in sexual development is supported by the reported role of its orthologues from *S. pombe* (STE11) and *P. anserina* (PaHMG5).

On the basis of the orthologous relation of HmbA, HmbB and HmbC proteins with the *P. anserina* PaHMG6, mtHMG1 and PaHMG4 proteins, respectively, we compared the role of the orthologous proteins to assess functional relations. The orthologue of HmbA in *P. anserina*, PaHMG6, is required for achieving normal-sized colony. This is a qualitatively similar function that was seen in the case of HmbA of *A. nidulans*. In a homozygous cross, the *Pahmg6Δ* mutant produced fruiting bodies with 50 times less abundance, with smaller body and larger neck than the wild type and began to eject ascospores several days later than the wild type. Although both *A. nidulans hmbAΔ* and *P. anserina Pahmg6Δ* mutants showed a delay in the time-course of sexual development in homozygotic crosses, an analogy cannot be draw between their functions in the aspect of sexual competency.

Absence of the orthologue of HmbB in *P. anserina AS1*$^{+}$ strain (*mthmg1Δ, AS1*$^{+}$) does not result in the loss of germination ability of the ascospores (ascospores germinate slowly with a spindly phenotype, thus we cannot draw an analogy between the functions of mtHMG1 and HmbB. Although the dual localization of mtHMG1 protein of *P. anserina* was not studied yet, we previously revealed a dual localization of HmbB and found that the orthologous HmbB and mtHMG1 share a third HMG-box domain, called Shadow-HMG-box, which is characteristic to the HmbB orthologues across Pezizomycotina. The structural similarity between HmbB and mtHMG1 and the fact that both proteins modulate the expression of nuclear genes makes it reasonable to suppose that mtHMG1 fulfills nuclear-localization-linked functions as we suggested previously for HmbB.

The HmbC counterpart in *P. anserina*, PaHMG4, was required for the normal distribution of the fruiting bodies. The PaHMG4 deletion mutant produced five times more spermatia (with wild type-like viability), whereas the deletion had no effect on female fertility. The PaHMG4 functions differ from that of HmbC, thereby the two proteins are functionally diverged.

Some of the physiological functions of HMGB proteins we revealed are specific for *A. nidulans* compared to yeast and *P. anserina*. This includes that HmbA and HmbB play a role in sensing of and/or response to environmental signals. By revealing the functional connections of HmbA and HmbB with signal transduction pathways, one would gain a deeper understanding of the regulatory machinery that governs physiological responses to environmental changes. On the other hand, we found that HmbC functionally interacts with VeA, a key regulator of the coordination of asexual and sexual development, as well as of secondary metabolism. By revealing the functional interactions of HmbC, one would gain a deeper insight into the regulation of these biological processes. Finally, HmbA, HmbB and HmbC are equally important in the positive regulation of mating-type genes, and thereby have a great impact on ascospore production in *A. nidulans*. The knowledge on the regulation of fungal mating-type genes is scarce, thereby clarifying, whether these HMGB proteins influence *matA*/*matB* expression directly or indirectly (e.g. via the modulation of upstream regulatory factors) would be of great interest. Additionally, future works should elucidate the gene-expression modulatory role of the HMGB proteins on a genome-scale that might lead to a more detailed characterization of the physiological roles of HmbA, HmbB and HmbC.

Intracolonial localization of STC production *in vivo*

The role of STC as protective agent against arthropod fungivores has been well established and the heterogeneous distribution of STC in the colony in association with the sexual structures was proposed previously. In addition, studies on sexual development and STC biosynthesis revealed that these processes share common master regulators and thereby they are coupled in environmental conditions that allow their manifestation. Here we show that StcO plays an essential role in STC biosynthesis and the activation of the *stcO* gene is an indicator of STC production at the third day of incubation. At the fourth day of incubation, we did not detect any *stcO* promoter activity, however the amount of STC further increased in comparison to the amount of STC detected at the third day. The *stcO* activated hyphae could be seen only in the proximity of groups of Hülle cells (forming primordia) and the promoter activity diminished with the distance from the Hülle cells. Mutants (e.g., *sfaDΔ*) with abolished cleistothecia formation and normal Hülle cells show no sign of STC production, indicating that the presence of Hülle cells is not deterministic for STC production. Notably, STC is normally produced in liquid cultures where sexual structures (including Hülle cells) are not formed. Uncoupling of these two processes might be governed by a variety of signal transduction pathways, which transduce and transmit environmental signals that determine sexual development and STC production. Sexual development depends on the MAP kinase pathways, while STC production relies on both MAP kinase- and PKA pathways, the latter being repressive on STC production. Therefore it is possible that while MAP kinase pathway does not support sexual development, repression of PKA pathway might be relieved and allow AflR activation and subsequent STC production. According to the abovementioned phenomena together with our results, we propose that on solid medium STC production is defined by (an) early sexual developmental factor(s) that locally initiates sexual development. Moreover, according to the

observed pattern of the *stcO* promoter activity, the promoting factors(s)/signal(s) of STC production can be subsequently transmitted to the vegetative hyphae located close to the core of sexual development. The lack of activity in distant hyphae indicates that the vegetative mycelium might consist of morphologically uniform, but functionally different hypha cells. Future works should elucidate the underlying mechanism of signal transmission for STC production from the origin to the nearby vegetative hyphal compartments.

SUMMARY

Our scientific results that carry novelty are the following:

- The HMGB proteins HmbA, HmbB and HmbC, are pivotal for the production and viability of ascospores.
- HmbC functionally interacts with VeA, the key regulator of development and secondary metabolite production.
- HmbA and HmbB plays role in the sensing and transmission of and/or response to environmental signals.
- HMGB proteins of *A. nidulans* influence the transcription of sexual development involved regulator genes. They inhibit the expression of 3 genes and activate the expression of 13 genes.
- All three HMGB proteins, HmbA, HmbB and HmbC are essential for the normal transcription of mating-type factor genes (*matA* and *matB*) via either direct regulation and/or indirect regulation (e.g. by modulating upstream regulatory functions).
- StcO is essential for the sterigmatocystin biosynthesis.
- Sterigmatocystin is produced only on those vegetative hyphal compartments, which are in the close proximity of the core of sexual differentiation.

- We propose that on solid medium sterigmatocystin production is defined by (an) early sexual developmental factor(s) that locally initiates sexual development.
- We propose that the promoting factors(s)/signal(s) of sterigmatocystin production can be subsequently transmitted to the vegetative hyphae located close to the core of sexual development

PUBLICATIONS

Publications in referred journals summarizing the results of this Ph.D. Thesis

Bokor E, Ámon J, **Keisham K**, Karácsony Z, Vágvölgyi C, Hamari Z. (2019) HMGB proteins are required for sexual development in *Aspergillus nidulans*. *PLoS One*. 14(4):e0216094. doi: 10.1371/journal.pone.0216094. eCollection 2019. PMID: 31022275
Impact Factor in 2018: 2.7666

Ámon J, **Keisham K***, Bokor E, Kelemen E, Vágvölgyi C, Hamari Z. (2018) Sterigmatocystin production is restricted to hyphae located in the proximity of hülle cells. *Journal of Basic Microbiology* 58(7): 590-596.
Impact Factor in the year of publication: 1.58
***joined first author**

Cumulative impact factor: 4.3466

DECLARATION OF CO-AUTHORS

I, the undersigned, declare that contribution of Keisham Kabichandra Singh to the results of the below listed publications was deterministic and pivotal.

Bokor E, Ámon J, **Keisham K**, Karácsony Z, Vágvölgyi C, Hamari Z. (2019) HMGB proteins are required for sexual development in *Aspergillus nidulans*. *PLoS One*. 14(4):e0216094.

Ámon J, **Keisham K***, Bokor E, Kelemen E, Vágvölgyi C, Hamari Z. (2018) Sterigmatocystin production is restricted to hyphae located in the proximity of hülle cells. *Journal of Basic Microbiology* 58(7): 590-596.

***joined first author**

The results published in these publications were not and will not be used in the past or in the future, respectively, for the purpose of acquiring an academic degree or title.

.....

Dr. Zsuzsanna Hamari