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

Study on the AtfA and AtfB bZIP-type transcription factors in the filamentous fungus *Aspergillus nidulans*

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1. Introduction

According to János Selye, the stress is a set of non-specific effects (for example, disease-causing or drug-induced effects) on a living organism. The stressors are all factors that can be characterized by the ability to cause stress (Selye, 1973). The environmental stress also affects the life cycle of microorganisms, however these microbes can overcome these effects by developing different strategies. Under stress they can adapt to environmental changes by activating specific signal transduction pathways through the activation of some transcription factors (TFs) (Guan et al., 2017). Therefore, regulation of signaling pathways plays a fundamental role in maintaining the cellular homeostasis of microorganisms. TFs are important elements of these regulatory networks (Leiter et al., 2021).

In fungi, the number of TF proteins, whose function is experimentally proven, is significantly less than in higher eukaryotes, this is also clearly visible in the TRANSFAC (http://gene-regulation.com/pub/databases.html) and Mycopath (https://www.biobase.cc) databases. This fact can be explained in two ways: since the idea is logical that the more complex an organism is, the more TFs it needs, therefore, it is possible that fungi can indeed have less TFs. According to another possibility fungi have a large number of TFs, but they have not yet been identified (Shelest, 2008).

bZIP (basic leucine zipper) type TFs are among the most ancient TFs that evolved from a single eukaryotic gene during evolution (Leiter et al., 2021). One of the best-known TF in humans is ATF2, which regulates important processes such as the cell cycle, glycosylation, inflammation, or the response to amino acid starvation (Watson et al., 2017). In fungi, the first ATF2 orthologue was described in *Schizosaccharomyces pombe* as Atf1 (Takeda et al., 1995). These bZIPs show great diversity in their function, they are involved in the regulation of many biological processes, such as vegetative growth, sporulation, germination, virulence and responses to abiotic and biotic stress (Zhao et al., 2022). We studied the AtfA and AtfB transcription factors belonging to the bZIP TF family and providing protection against oxidative stress in conidia and vegetative mycelium in many filamentous fungi (Balázs et al.; 2010, Roze et al., 2011, Hagiwara et al.; 2014, Silva et al., 2021).

AtfA is involved in the transcriptional regulation of stress responses, contributes to the maintenance of the viability of conidia, is involved in conidia's tolerance to oxidative, heat (Hagiwara et al., 2008) and osmotic stress (Balázs et al., 2010). It also plays a role in asexual development in *Aspergillus nidulans* and in maintaining the dormancy of conidia in *Fusarium verticillioides* (Szabó et al., 2020). Under oxidative stress, it also regulates several primary

metabolic pathways (amino acid, fatty acid and tricarboxylic acid metabolic processes) in *A. nidulans* (Orosz és mtsai., 2017).

In *Aspergillus oryzae* AtfB provides protection against oxidative (Wee et al., 2017) and heat stress in the conidiospores, regulates cellular processes such as tolerance to various stress or carbon and amino acid metabolism during the development of conidia (Sakamoto et al., 2008, Silva et al., 2021). AtfB is a master regulator of secondary metabolism, stress response, and conidial development in *Aspergillus parasiticus* (Wee et al., 2017).

In my doctoral work, we performed phenotypic studies of the deletion and overexpression mutants of *atfA* and *atfB*, as well as transcriptomic analysis of the deletion mutants and a control strain to map the additional functions of the *atfA* and *atfB* genes encoding bZIP transcription factors in *A. nidulans*.

2. Objectives

In my doctoral work, we aimed the following studies in order to gain a deeper understanding of the functions of the *atfA* and *atfB* genes encoding bZIP-type transcription factors in the filamentous fungus *A. nidulans***:**

- Generation of the deletion mutants (*Δ*) with Double-Joint PCR method and construction of the overexpression (OE) mutants with the pHS11 vector including *niiA* promoter in all combination: *ΔatfA, ΔatfB, ΔatfAΔatfB, ΔatfAatfBOE, ΔatfBatfAOE, atfAOE, atfBOE* és *atfAOEatfBOE,*
- Testing the stress sensitivity of mutants by point-inoculation on solid medium using oxidative (diamide, MSB, *t*BOOH), osmotic (NaCl, sorbitol), heavy metal ($CdCl_2$), cell wall integrity stress (Congo red) and testing the heat sensitivity of conidiospores at 50 °C for 10 min,
- Studying sexual (by inducing the formation of cleistothecium) and asexual (by determining the number of conidiospores) reproduction,
- Determination of the size of conidiospores using light and scanning electron microscopy and the *abaA* gene expression by RT-qPCR,
- The production of secondary metabolites is closely related to environmental, mainly oxidative stress, so we aimed to analyze sterigmatocystin mycotoxin production by thin layer chromatography and HPLC,
- Transcriptomic study of the deletion mutants (*ΔatfA*, *ΔatfB*, *ΔatfAΔatfB*) and the control strain (THS30.3) by next-generation RNA sequencing of mycelium and conidial samples from surface cultures in the presence and absence of MSB,
- Then we aimed to perform a comparative transcriptome analysis of the data and to evaluate the transcriptomic results by Venn diagram comparisons of the global gene expression patterns collected from all strains.

3. Materials and methods

Deletion of the *atfA* (locus ID: AN2911) and *atfB* (locus ID: AN8643) genes was performed by the Double-Joint PCR method (Yu et al., 2004; Leiter et al., 2016). Deletion mutants were constructed by Dr. Mi-Kyung Lee (Korea Bioscience and Biotechnology Research Institute (KRIBB), Biological Resource Center, Jeongeup-si, Republic of Korea).

The pHS11 vector containing three quarters part of the coding sequence of the *pyroA* marker gene was used to generate the overexpression mutants (Kwon et al., 2010). The recipient strains to be transformed with this vector show auxotrophy to pyridoxine due to mutation. The insertion of the plasmid containing *atfA* or *atfB* into the *pyroA4* site by single recombination rescues the pyridoxine auxotrophy. The *atfA* and *atfB* genes were cloned between the *niiA* promoter and *trpC* terminator (Leiter et al., 2016).

In the overexpression mutants, insertion of *atfA* or *atfB* was checked by PCR (EmeraldAmp MAX PCR polymerase, Takarabio), and overexpression was determined by RT-qPCR method (Király et al., 2020).

Stress sensitivity assays

5 μl (100,000 spores) of spore suspension with a spore concentration of $2X10^7$/ml from surface cultures incubated on minimal medium for 6 days at 37 ^oC was point-inoculated onto medium containing various stress-generating agents and strains were incubated at 37 ^oC for 5 days (Balázs et al., 2010). THS30.3 strain was used as a control strain.

Oxidative stress was triggered with 2 mM Diamide, 0.08 mM MSB and 0.8 mM *t*BOOH, osmotic stress with 1.5 M NaCl and 2 M sorbitol, heavy metal stress with 300 μM CdCl2 and cell wall integrity stress with 54 μM Congo Red. We also tested the viability of conidiospores under heat stress at 50 ^oC for 10 minutes (Hagiwara et al., 2008, 2009).

Evaluation of the physiological characteristics of the mutants

In the sexual reproduction studies, cleistothecium formation was induced. Mature cleistothecia on the Petri-dishes were counted using a stereomicroscope and their quantity was determined by normalization to the area (cm^2) (Leiter et al., 2016).

In the asexual reproduction tests, to study the conidiospore formation of the mutant and control strains, the spores were collected in 3 ml of spore washing solution, by washing the whole colony, counted under a Bürker chamber and the amount of conidiospores per cm^2 was determined (Vargas-Pérez et al., 2007).

To assess the size of the conidiospores by taking light and scanning electron microscopic images, we measured the spore diameters with ToupView image processing software. The electron microscope images were photographed by Dr. Lajos Daróczi, a colleague at the Department of Solid State Physics, Institute of Physics, Faculty of Science and Technology, University of Debrecen. The expression of the *abaA* gene (locus ID: AN0422) regulating the development of conidiophores was determined by RT-qPCR in the control strain and in *ΔatfB* and *atfB*OE mutants, Dr. Gyula Batta from the Institute of Biotechnology, Department of Genetics and Applied Microbiology, Faculty of Science and Technology, University of Debrecen, assisted in the measurements and then we evaluated the collected data.

The sterigmatocystin producing ability of the mutants was analyzed from point-inoculated cultures (Yin et al., 2013) by thin layer chromatography followed by HPLC analysis. The measurements were carried out by Dr. Tibor Nagy, a colleague of the Department of Applied Chemistry, Institute of Chemistry, Faculty of Science and Technology, University of Debrecen.

Transcriptomic analysis of the deletion strains and a control strain by next-generation RNA sequencing

In the Genomic Medicine and Bioinformatics Service Laboratory at the University of Debrecen using the Ilumina NextSeq500 device we carried out a transcriptomic study of the mycelium and conidiospore morphological states of the deletion mutants, *ΔatfA*, *ΔatfB*, *ΔatfAΔatfB* and the control strain treated with and without MSB at a concentration of 0.04 mM. The relative expression levels were determined by comparing the RPKM values (Reads Per Kilobase of gene model per Million mapped reads) calculated to show the transcriptional activity of the genes (Robinson et al., 2010). The differentially expressed genes (DEG) were determined by Dr. Károly Antal (Eszterházy Károly University, Department of Zoology).

The transcriptomic data were grouped according to the tested strain, treatment and developmental state. The evaluation of the transcriptomic data was carried out by Dr. Károly Antal (Eszterházy Károly University, Department of Zoology) and Prof. Dr. Tamás Emri (the colleague of the Department of Molecular Biotechnology and Microbiology (University of Debrecen and the HUN-REN-DE Fungi Stress Biology Research Group).

We generated the following gene categories to evaluate the data: AA genes are regulated only by the *atfA* gene, BB genes are regulated only by the *atfB* gene, for AB genes, both genes (*atfA* and *atfB*) are required together for the normal function of the gene, for A/B genes regulation of both genes are required and the missing TF can be completely replaced by the

other TF, while in the case of A-B genes both genes are required but the missing TF can only be partially replaced by the other.

In the gene set enrichment analysis (GSEA) genes were ranked by the correlation of the expression differences between them (Subramanian et al., 2005).

The experimental results were given by the mean and standard deviation of three independent measurements. The differences between the mean values were determined with Student's t-test, where only $p<0.05$ was considered statistically significant.

The relative transcript levels of *atfA* and *atfB* genes were calculated by determining the value of ΔC_P ($\Delta C_P = C_{P\text{reference}} - C_{P\text{tested gene}}$) (Balázs et al., 2010). The *actA* gene (locus ID: AN6542) was used for normalization.

4. Summary of the new scientific results

bZIP transcription factors are important regulators of various developmental processes, stress responses and secondary metabolite production in filamentous fungi (Kocsis et al., 2022; Roze et al., 2011; Yin et al., 2012, 2013; Bákány et al., 2021).

The aim of this thesis was the functional analysis of the *atfA* and *atfB* genes encoding bZIP-type transcription factors of the filamentous fungus *A. nidulans*, and the transcriptomic study of the deletion mutants (*ΔatfA*, *ΔatfB*, *ΔatfAΔatfB*) by next-generation RNA sequencing. To analyze the physiological functions of the *atfA* and *atfB* genes, we generated the deletion and overexpression mutants in all combinations, namely: *ΔatfA*, *ΔatfB*, *ΔatfAΔatfB*, *ΔatfAatfBOE, ΔatfBatfAOE, atfAOE, atfBOE* and *atfAOEatfBOE*.

1. The results of the oxidative, osmotic, heavy metal, cell wall integrity and heat stress studies

1.1. *ΔatfA, atfB*OE and *atfA*OE*atfB*OE strains showed reduced growth on minimal medium at 37 °C.

1.2. In the *ΔatfA* and *ΔatfAatfBOE* strain and the double deletion strain we observed sensitivity to diamide, whereas the deletion and overexpression of *atfB* caused a diamide tolerant phenotype.

1.3. The role of AtfA in the protection against oxidative stress has already been confirmed previously (Hagiwara et al., 2008, 2009; Balázs et al., 2010; Emri et al., 2015), in our experiments in addition to *ΔatfA*, we also observed *t*BOOH sensitivity in the double deletion and *ΔatfAatfB*OE mutants, whereas the *atfA*OE mutant showed increased tolerance to *t*BOOH.

1.4. Only the *ΔatfA* mutant showed sensitivity to MSB, which was compensated by overexpression of *atfB* in the *ΔatfAatfB*OEmutant. a*tfA*OE and *atfB*OE mutants grow better in the presence of MSB than the control strain.

1.5. The osmotic stress sensitivity of the mutants was tested in the presence of NaCl and sorbitol, with the result that the deletion of *atfB* caused sensitivity to NaCl, while caused tolerance in the double overexpression strain. The *atfB*OE strain was the most sensitive to sorbitol, while the *ΔatfB* tolerated this stress generating agent well.

1.6. Our heavy metal stress studies revealed that AtfB plays a role in $CdCl_2$ stress, since as a result of *atfB* overexpression a mild sensitivity was observed in the *ΔatfAatfB*OE mutant, while a more significant sensitivity was observed in the *atfB*OE and *atfA*OE*atfB*OE strains. The *ΔatfB* strain showed moderate tolerance to $CdCl_2$.

1.7. The *ΔatfA* strain showed moderate tolerance to cell wall integrity stress tested with Congo Red.

1.8. The role of AtfB in heat stress was also demonstrated, since the viability of the *ΔatfB* mutant was reduced, on the other hand, the viability of the *ΔatfAatfB*OE and *ΔatfBatfA*OE mutants increased after heat treatment at 50 ^{o}C for 10 minutes.

2. The results of experiments investigating sexual and asexual reproduction as well as sterigmatocystin production

2.1. The bZIP transcription factors are also involved in sexual reproduction in filamentous fungi (Bayram et al., 2008; Yin et al., 2013). Our experimental results confirmed this previous finding since the deletion of *atfA* alone or together with the deletion of *atfB* inhibited cleistothecium formation, contrarily, *ΔatfB* and *ΔatfBatfA*OE mutants produced one and a half times more fruiting bodies compared to the control.

2.2. To evaluate asexual reproduction we also determined the number of conidiospores of the mutants. The number of conidiospores decreased in *ΔatfA*, *ΔatfAΔatfB*, *atfB*OE and *ΔatfAatfB*OE mutants, and increased in *atfA*OE and *ΔatfBatfA*OE.

2.3. By taking light and scanning electron microscopic images, we examined the size of the conidiospores with ToupView image processing software. The size of the spores of the *atfB*OE mutant was approximately around 3.72± 0.24μM (the spores of the control strain have a size of 2,98 ± 0,33 μM), which proved to be a significant difference. There was no difference in the spore size of the other mutants compared to that of the control strain. Considering the difference in spore size of *atfB*OE, the expression of the *abaA* gene (locus ID: AN0422, regulatory element of the central pathway of conidiogenesis) was determined by RT-qPCR from point-inoculated surface cultures. According to the RT-qPCR data, the *abaA* gene was upregulated in the *atfB*OE mutant, but in the *ΔatfB* mutant there was no significant difference compared to the control strain.

2.4. We also determined the level of sterigmatocystin production. According to our HPLC analysis, sterigmatocystin level was below the limit of detection in the *ΔatfA* mutant after 5 days incubation. In contrast to this, this mycotoxin-producing ability of the double deleted, *ΔatfAΔatfB* mutant was not altered at all. The *ΔatfAatfB*OE, *ΔatfBatfA*OE and *atfA*OE*atfB*OE strains were also able to produce smaller amount of sterigmatocystin. The sterigmatocystin level of the double overexpression, *atfA*OE*atfB*OE mutant was halved compared to the control strain.

2.5. The link between sexual reproduction and secondary metabolism was confirmed by the fact that in the *ΔatfA* mutant inhibition of sterigmatocystin synthesis occurred concomitantly with the disturbance of sexual reproduction.

3. Our transcriptomic results were as follows

3.1. The expression of the *atfA* gene did not change either by MSB treatment or *atfB* deletion.

3.2. The *atfB* gene was repressed in mycelium samples after MSB treatment and in mycelium and conidia samples after the *atfA* deletion, consequently AtfA can regulate AtfB-dependent genes through the transcription of *atfB.*

3.3. In both mycelium and conidial samples, *ΔatfA* was found to have a much higher amount of differentially expressed genes compared to *ΔatfB*.

3.4. The number of AtfA-dependent genes (329) in untreated mycelium samples was much higher than that of the AtfB-dependent genes (96), most of which were also AtfA-dependent, therefore, the absence of the *atfA* gene has more significant transcriptomic and physiological effect on *A. nidulans* (Kocsis et al., 2022).

3.5. Fewer genes showed transcriptional changes upon MSB treatment due to *atfA* and/or *atfB* deletion. In the control strain, MSB treatment resulted in the repression of *atfB.*

3.6. In the MSB-treated cultures, the AtfA-dependent gene set was characterized by the enrichment of downregulated genes sensitive to MSB stress, similar to the untreated AtfA- and AtfB-dependent gene sets. In cultures showing an MSB tolerant phenotype, the main role of AtfA was to inhibit further downregulation of genes.

3.7. The MSB treatment also significantly modified the transcriptome of conidia and mycelia (in the conidium: 485 up-regulated genes and 1070 down-regulated MSB stress-sensitive genes, in the mycelium: 786 up-regulated and 912 down-regulated MSB-sensitive genes were found).

3.8. The AtfA-dependent genes identified by Gene Set Enrichment Analysis were as follows: genes involved in carbohydrate metabolism, glycolytic genes (in conidiospores), genes regulating phosphorelay response (except in untreated mycelium samples), iron-sulfur cluster assembly genes (in conidiospores), genes encoding antioxidant enzymes (in untreated conidiospore cultures), tricarboxylic acid cycle (TCA cycle) and respiratory chain genes (in MSB-treated conidiospore cultures), secondary metabolite cluster genes (emericellamide cluster).

3.9. There was a greater difference between AtfA-dependent gene groups in vegetative mycelia compared to conidia. We assume that conidia can adapt to MSB during conidiogeneis.

Conidiogen cells alter the mRNA content of conidia to prepare them for the stresses most likely occuring during germination.

3.10. Only 23 AtfB-dependent genes were found exclusively, for example AN8953 (*agdB*) a putative α-glycosidase, AN3402 (*amyB*) a putative α-amylase, AN7619 (*calA*) plays a role in early conidial germination, and the gene AN2099, which encodes an alternative oxidase (Kocsis et al., 2022)

3.11. The *atfA* and *atfB* mRNA levels were much higher in conidiospores, in addition, AtfA- and AtfB-dependent genes were also more abundant in this developmental stage, so these genes have a more important regulatory role in spores than in vegetative tissue (the important regulatory role of AtfA in conidia has already been confirmed in other *Aspergillus* species, AtfA plays a significant role in the regulation of conidia-specific genes).

4. Processes presumably regulated by AtfA and AtfB are the following:

4.1. Protection against heat stress: The *ΔatfAatfB*OE, *ΔatfBatfA*OE mutants showed the most significant tolerance to heat stress.

4.2. Cleistothecium formation: In the *ΔatfA and ΔatfAΔatfB* strains sexual reproduction failed, in contrast to this the *ΔatfBatfA*OE strain produced a similar amount of cleistothecia as the *ΔatfB* mutant, and in the two latter mutants, the number of cleistothecia was significantly higher than in the control.

4.3. Conidiospore formation: Both the deletion of *atfA* and the overexpression of *atfB* reduced the number of conidia. Overexpression of *atfA* alone or in combination with the deletion of the *atfB* gene increased the number of conidiospores,

4.4. ST production: The ST production of *ΔatfA* remained below the detection limit, while the ST production of the double overexpression mutant as well as the *ΔatfAatfB*OE, *ΔatfBatfA*OE mutants also decreased compared to the control.

5. Summary

The dimeric basic leucine zipper (bZIP) proteins are conserved transcriptional enhancers found in all eukaryotes. They play a critical, often species-specific role in many aspects of the development of an organism (Yin et al., 2013). In eukaryotes, they play a diverse role in the differentiation and maintenance of cell types, but are also involved in various stress responses, regulation of cell proliferation and maintenance of homeostasis (Jindrich and Degnan, 2016).

Our experiments confirmed that AtfA is involved in the defense against oxidative stress, and we also proved that it also plays an important role in sexual reproduction, its deletion (alone or together with the deletion of *atf*B), caused the inhibition of cleistothecia formation.

The close link between sexual reproduction and secondary metabolism was demonstrated by the fact that disturbances in the sexual reproduction coincided with the failure of the mycotoxin sterigmatocystin synthesis in *ΔatfA*.

Regarding the function of AtfB, it is elucidated that it mainly plays a role in the protection against heat and heavy metal stress, it also coordinates the development of conidiospores, by influencing the amount of conidia.

Our results also shed light on that certain physiological functions are regulated by both bZIP transcription factors, for instance while studying heat stress, sexual reproduction, formation of conidia and sterigmatocystin production.

A comparative analysis of the transcriptome of the control and deletion strains showed that by deletion of *atfA*, encoding the primary bZIP TF in the stress responses of *A. nidulans*, a much larger number of down- and up-regulated genes were found in both the mycelium and conidium samples, and that AtfB-dependent genes were also characterized by the dependence on AtfA.

Examining the two developmental stages we could conclude that the conidial samples had much higher levels of *atfA* and *atfB* mRNA and we were also able to identify more AtfA- and AtfB-dependent genes. As a result of the treatment with MSB, the number of only AtfB dependent genes decreased significantly, bringing about a decrease in the mRNA level of the MSB-treated mycelium sample of the control and the *ΔatfA* mutant conidia. We found that the overlap between AtfA-dependent genes of MSB-treated and untreated samples was low in mycelial samples.

Gene expression analysis unfolded that the *atfA* and *atfB* genes have cell type-dependent regulatory roles in the studied filamentous fungi *A. nidulans*, and that AtfA plays a more important role as a component of the regulatory network than AtfB.

Summing it up, according to our results there is probably an interaction between AtfA and AtfB that can regulate the expression of genes involved in stress tolerance, sexual and asexual reproduction and secondary metabolite production. Therefore, our further research plan is to demonstrate this putative interaction. We are planning to determine the AtfA and AtfB binding sites on the promoters at the genome level by chromatin immunoprecipitation sequencing (ChIP-Seq) and to confirm AtfA-AtfB heterodimer formation by bimolecular fluorescence complementation (BiFC) technique.

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7. Publications on which the PhD thesis is based

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Candidate: Beatrix Kocsis
Doctoral School: Doctoral School of Pharmacy
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List of publications related to the dissertation

1. **Kocsis, B.**, Mi-Kyung, L., Antal, K., Jae-Hyuk, Y., Pócsi, I., Leiter, É., Emri, T.: Genome-Wide Gene Expression Analyses of the AtfA/AtfB-Mediated Menadione Stress Response in Aspergillus nidulans.
Cells. 12 (3), 1-16, 2023.
DOI: https://doi.org/10.3390/cells12030463
IF: 6 (2022)

2. **Kocsis, B.**, Lee, M. K., Yu, J. H., Nagy, T., Daróczi, L., Batta, G., Pócsi, I., Leiter, É.: Functional analysis of the bZIP-type transcription factors AtfA and AtfB in Aspergillus nidulans.
Front. Microbiol. 13, 1-9, 2022.
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3. Selbmann, L., Benkő, Z., Coleine, C., de Hoog, S., Donati, C., Druzhinina, I. S., Emri, T., Ettinger, C. L., Gladfelter, A. S., Gorbushina, A., Grigoriev, I. V., Grube, M., Gunde-Cimerman, N., Karányi, Z., **Kocsis, B.**, Kubressoian, T., Miklós, I., Miskei, M., Muggia, L., Northen, T., Novak-Babič, M., Pennacchio, C., Pfliegler, V. P., Pócsi, I., Prigione, V., Riquelme, M., Segata, N., Schumacher, J., Shelest, E., Sterflinger, K., Tesei, D., U'Ren, J. M., Varese, G. C., Vázquez-Campos, X., Vicente, V. A., Souza, E. M., Zalar, P., Walker, A. K., Stajich, J. E.: Shed Light in the DaRk LineagES of the Fungal Tree of Life-STRES.
Life (Basel). 10 (12), 1-13, 2020.
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Total IF of journals (all publications): 15,017
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