PhD Thesis

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Functional analysis of *FvatfA* and *FvmnSOD* genes involved in the oxidative stress response in *Fusarium verticillioides*

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Background and the aims of investigation

F. verticillioides is a worldwide pathogen of maize, which can be associated with mould infections of stem and corncob and also can cause root rot on seedlings. It produces several secondary metabolites, including fumonisins, of which fumonisin B1 (FB1) and fumonisin B2 (FB2) are the most abundant (Blacutt *et al.*, 2018). Fumonisins can contaminate both animal feed and food for human consumption (Logrieco *et al.*, 2002; Covarelli *et al.*, 2012).

Fungi respond to oxidative stress through defense mechanisms induced via different signaling pathways. In yeast, external stimuli mainly activate mitogen-activated protein kinase (MAPK) signal transduction pathways, which are evolutionarily conserved in filamentous fungi as well. Transcription factors activated via the MAPK pathways coordinate the level and timing of the expression of target genes, such as antioxidant enzymes, and genes involved in the biosynthesis of secondary metabolites, thus regulating metabolic processes and the cellular stress response. In Aspergilli, antioxidant enzymes represent the primary line of defense against oxidative stress, while secondary metabolites belong to the secondary line of defense (Reverberi *et al.*, 2006; Hong *et al.*, 2013b).

The structure of the bZIP-type transcription factors belonging to the CREB/ATF protein family (basic motif in the DNA binding region and leucine zipper for dimerization) and their regulatory mechanisms are highly conserved in yeasts and filamentous fungi. One of the best characterized bZIP transcription factors is *Schizosaccharomyces pombe* Atf1 protein, which is a homolog of human ATF2. Amongst the target genes of Atf1, there are a number of environmental stress response genes (Chen *et al.*, 2003; Yaakoub *et al.*, 2022). The functions of several orthologs of the Atf1 bZIP-type transcription factor have already been studied in various filamentous fungal species (Leiter *et al.*, 2021).

Our research focused on *FvatfA* encoding the *S. pombe* Atf1 orthologue FvAtfA bZIP-type transcription factor activated by Sty1 MAPK, and the *FvmnSOD* gene encoding the mitochondrial manganese superoxide dismutase (MnSOD) enzyme; we investigated the role of these genes play in the growth, stress sensitivity and production of secondary metabolites of the fungus.

The main goal of the PhD work were as follows:

1. Identification of the *FvatfA* gene using *in silico* methods in *F. verticillioides.*
2. Generating the *ΔFvatfA* deletion mutant using the double joint PCR method and complementation of the null mutant with the wild-type *FvatfA* gene.
3. Clarification of its role
    - in vegetative growth and sporulation, as well as in the viability and size of spores;
    - in invasive growth and sexual reproduction;
    - in abiotic (osmotic, oxidative, heavy metal, cell wall) stress sensitivity;
    - in the production of secondary metabolites, such as fumonisins, bikaverin and carotenoids.
4. Identification of the *FvmnSOD* gene using *in silico* methods in *F. verticillioides.*
5. Generating the *ΔFvmnSOD* deletion mutant using the double joint PCR method and complementation of the null mutant with the wild-type *FvmnSOD* gene.
6. Clarification of its role
    - in vegetative growth;
    - in invasive growth and sexual reproduction;
    - in abiotic (osmotic, oxidative, heavy metal, cell wall) stress sensitivity, as well as in apoptosis induced by PAF;
    - in the production of fumonisins;
    - in the morphology and function of mitochondria.

Materials and methods

Origin, maintenance and culture of the strains used

The wild-type reference strain *F. verticillioides* FGSC 7600 (Fungal Genetics Stock Center Kansas City, MO, USA) was used to create the *ΔFvatfA* and *ΔFvmnSOD* deletion mutants. Ectopic complemented strains were also created from the deletion mutants. The conidiospore suspensions of the strains were stored in 50% glycerol at -70 °C.

Preparation of gene deletion and complementation constructs

The gene deletion cassettes were created using double-joint PCR method (Sagaram *et al.*, 2007; Yu *et al.*, 2004). In the first step, the 5' and 3' flanking regions were amplified from the genomic DNA isolated from the wild-type strain *F. verticillioides* FGSC 7600 using Expand™ Long Polymerase (Roche). Simultaneously, the hygromycin B phosphotransferase-encoding gene (*hph*) was amplified from the plasmid vector pBP15 (Sagaram *et al.*, 2007). Subsequently, in the second step (joint PCR), the three purified PCR products were mixed together in a tube in 1:3:1 molar ratio and joined by PCR using the overlapping regions. Finally, in nested PCR the gene deletion cassettes containing the marker gene were amplified. The resulting PCR products were purified.

The *ΔFvatfA* and *ΔFvmnSOD* strains were complemented with the wild-type *FvatfA* and *FvmnSOD* genes, to which the aminoglycoside 3'-phosphotransferase gene (*gen*) responsible for geneticin resistance was fused using single-joint PCR (Sagaram *et al.*, 2007).

Transformation of *F. verticillioides* protoplasts

For the PEG-mediated transformation, *F. verticillioides* protoplasts were generated according to Sagaram *et al.* (2007). The protoplasts were layered on the surface of regeneration agar (which contained hygromycin B at a concentration of 100 μg/ml), and the plates were incubated at 28 °C for 7-15 days, until the appearance of transformant colonies.

Southern blot analysis

The single, correct insertion of the deletion cassette was confirmed by Southern blot analysis, for which the genomic DNA isolated from wild-type and transformant colonies was digested with restriction endonuclease at 37 °C, and then the samples were run on a 0.7% agarose gel. The DNA was transferred from

the gel to an Immobilon NY+ (Millipore) membrane using vacuum transfer. The membrane was developed according to the manufacturer's instructions of the DIG DNA detection and Labeling Kit (Roche).

Copy number determination

The copy number determination in the complemented strains was determined by quantitative real time PCR assay according to Herrera *et al.* (2009). Genomic DNA was isolated from the strains, and then a qPCR mixture was prepared using Fast SYBR® Green Master Mix. The copy number was determined using the method of absolute quantification.

Comparison of the growth of strains

To determine growth rate of the strains, Czapek-Dox or PDA agar plates were inoculated with 5 µl spore suspension (concentration of 2×10^7/ml), then plates were incubated for 6 days at 25 °C. At the end of the incubation period colony diameters were measured and a photograph was taken of the plates.

Determination of spore size, conidiation and spore viability

Microscopic pictures of spores washed from seven-day-old cultures grown on Czapek-Dox agar were taken (400×). The arc lengths and diameters of the spores were measured using the ImageJ software.

Conidiation was tested according to Shim *et al.* (2006), cell counting was done using a Bürker chamber.

Spore viability test was performed according to Choi and Xu (2010). Spores from seven-day-old Czapek-Dox cultures were washed with sterile distilled water after the concentration of the spore suspension was adjusted to 1×10^3/ml. The spore suspensions were incubated at 25, 42 and 45 °C or at 4 °C, then 100 µl of each suspension were plated on Czapek-Dox plates, and then colonies were counted.

Stress sensitivity tests

To test stress sensitivities, we supplemented the Czapek-Dox agar plates with one of the osmotic, oxidative, heavy metal and cell wall stress-generating agents at various concentrations. Plates were inoculated with 5 µl spore suspension (concentration of 2×10^7/ml). At the end of the incubation period colony diameters were measured and a photograph was taken of the plates.

Examination of the PAF sensitivity of the strains

The effect of *Penicillium chrysogenum* antifungal protein (PAF) was investigated as follows: PAF was added to Czapek-Dox agar to reach a final concentration of 100 or 200 μg/ml, then the medium was pipetted into individual wells of a 12-well plate. Each well was inoculated with 5 μl spore suspension (1×10^5 spore/ml) prepared from freshly washed microconidia. Plates were incubated at 25 °C for 72 hours. At the end of the incubation period colony diameters were measured and a photograph was taken of the plates.

Determination of the production of secondary metabolites

Measurement of fumonisin B1 and B2 production

After sterilization, 5 ml of the modified Myro medium (Han *et al.*, 2014) was measured into the wells of a six-well plate, then 50 μl of spore suspension of the tested strains (1×10^8 /ml) was added to each well. The cultures were incubated in a thermostat without illumination at 25 °C for two weeks in static condition. The mycelium from the surface cultures was collected at the end of the incubation period, measured into 1.5 ml centrifuge tubes of known weight, and then the dry mass was determined after lyophilization. The fumonisins were extracted from the lyophilized mycelia with a mixture of quartz sand and acetonitrile:water 50:50+1% formic acid, and then the fumonisin content of the samples was determined using a capillary electrophoresis mass spectrometry (Kecskeméti *et al.*, 2020). The obtained measurement results were normalized to the biomass and then averaged.

Measurement of bikaverin production

100 ml of bikaverin-inducing liquid media (Bell *et al.*, 2003) was inoculated with 1×10^8 spores and grown as shaken culture at 28 °C, 200 rpm for 5-7-9 days, respectively. At the incubation period the mycelium was collected by filtration and used for dry mass determination. The concentration of bikaverin in the supernatant was determined spectrophotometrically at 500 nm as described by Bell *et al.* (2003).

Measurement of carotenoid production

100 ml of liquid DG medium (Avalos *et al.*, 1985; Davis *et al.*, 1970; Hornero-Méndez *et al.*, 2018) were inoculated with 1×10^8 spores and grown as shaken culture at 25 °C, 150 rpm for 7 days under a light panel containing white light fluorescent tubes (at a light flux of 80 μmol photon m^{-2} s^{-1}). At the end of

incubation period the biomass of the cultures was determined as already described for bikaverin, then the remaining mycelium was collected in 15 ml centrifuge tubes, and then lyophilized. After acetone extraction from the lyophilized mycelia, the amount of carotenoids was determined using a spectrophotometer.

Measurement of gene expression

The expression of the genes involved in the biosynthesis of fumonisins, carotenoids and bikaverin was measured using RT-qPCR. The amount of RNA samples isolated from lyophilized mycelia was measured using NanoDrop and their quality was checked by agarose gel electrophoresis. Before RT-qPCR, the samples were denatured using DNase I Amplification Grade Kit (Sigma). For the RT-qPCR reaction Xceed qPCR SG 1-step Kit (IAB) was used, each reaction contained 500 ng of RNA. The expression level of the reference gene *tef1* (FVEG_02381) was also measured in each RNA sample. Relative expression levels were calculated using the 'delta method'.

Determination of invasive growth

Invasive growth was determined on tomato fruits (Di Pietro *et al.*, 2001). Agar discs cut out from cultures grown on Czapek-Dox agar were placed on the surface of tomato fruits. 72 and 96 hours after inoculation, the diameter of the colonies was measured.

Determination of sexual reproduction

The ability of the deletion and complemented strains to with the wild-type strain was tested on carrot agar (Klittich and Leslie, 1988). After crossing, the strains were incubated at 20/24 °C for 5-6 weeks, with a 12-hour dark period alternating with 12-hour light period provided by a white and a dark blue fluorescent lamp. *F. verticillioides* FGSC 7603 was used as a tester strain.

Determination of respiration

The respiration of the strains was measured from exponential growth phase mycelia of submerged cultures using an Oxigraph O_2 electrode (Oxylab 2, Hansatech Instruments Ltd, UK) at 25 °C. Total, cytochrome c-dependent KCN-sensitive, alternative oxidase-dependent (KCN-resistant, SHAM-sensitive) and residual (KCN+SHAM-resistant) respirations were determined. The cytochrome c-dependent pathway and alternative oxidase respirations were inhibited using KCN and SHAM (Leiter *et al.*, 2016; Medentsev *et al.*, 2002).

Examination of the morphology of mitochondria

The morphology of the mitochondria was examined with a confocal laser scanning microscope. In the three-channel Z-stack images were taken where the green channel showed mitochondria stained by MitoTracker Green, the red channel visualized the superoxide indicator dihydroethidium, whereas the blue channel (Calcofluor White staining) showed the structure of the hyphae and the boundaries for each segment. The volumetric ratio of mitochondria, relative superoxide ratio (the volume of superoxide {proportional to the ethidium signal formed} normalized to the percentage volume of the mitochondria), and the diameter of the second hyphal segment were determined according to Leiter *et al.* (2016).

Promoter analysis

In the sequences of the genes involved in the biosynthesis of fumonisins, bikaverin and carotenoids, we searched for possible ATF/CREB transcription factor binding sites in the promoter regions and in the non-transcribed 5' sections between the transcription start site and the translation start site (Roze *et al.*, 2011; Hong *et al.*, 2013a) using PROMO version 3.0.2.

Results

The *FvatfA* gene of *F. verticillioides*

Role of *FvatfA* in vegetative and invasive growth

The growth rate of the *ΔFvatfA* deletion strain was lower than that of the wild-type strain on both Czapek-Dox (-11.79%) and PDA (-22.16%) agar plates, and this phenotype was restored in the complemented strains.

The invasive growth of the strains was investigated on tomato fruits. In the case of the wild-type strain the average diameter of the colonies was 7.92 and 12.75 mm (after 72 and 96 hours, respectively), while the deletion mutant showed a significantly smaller growth: a diameter of the colonies were 2.25 and 3.08 mm. The percentage decrease in the growth rate on tomato fruits (-71.6 and -75.8%, after 72 and 96 hours) was much greater than was observed on Czapek-Dox and PDA agar plates.

Effect of *FvatfA* gene deletion on microconidia and sexual reproduction

The spore-producing capacity of the *ΔFvatfA* strain was significantly reduced compared to the wild-type strain: while the FGSC 7600 strain produced $6.21\pm0.29\times10^7$ spores per square centimeter, the *ΔFvatfA* mutant produced $3.98\pm0.36\times10^7$ (35, 9% less) spores under the same conditions.

The deletion mutant produced spores that were significantly shorter (8.07±0.13 μm) by almost 2 μm than the wild-type strain (10.43±0.23 μm). On the other hand, we did not observe any significant differences in the diameter of the spores between the two strains: the diameter of the spores of the wild-type parent strain was 4.85±0.66, while that of the spores of the *ΔFvatfA* mutant was 4.94±0.18 μm.

The viability of the conidiospores was tested by storing them at 42 or 45 °C for 60 minutes, and at 4 °C for 7 days, the spore suspensions were spread on the surface of Czapek-Dox agar plates; after 2 days of incubation the colonies were counted. Deletion of *FvatfA* did not affect spore viability under either heat or cold stresses.

Deletion of the *FvatfA* gene results in female sterility. The wild-type parent strain (FGSC 7600, MATA-1), the *ΔFvatfA* mutant, and the *FvatfA* 'C H7 and H9 complemented strains were used as female partners, and the FGSC 7603 (MATA-2) strain was used as a tester strain. After 40 days of incubation on carrot agar, we found that the *FvatfA* deletion mutant did not produce perithecia, while the wild-

type produced an average of 30 perithecia, the *FvatfA* 'C H7 strain 26, and the *FvatfA* 'C H9 strain 22 perithecia.

The role of *FvatfA* in the stress sensitivity of *F. verticillioides*

Orthologs of Atf1 (*S. pombe*) - AtfA (*A. nidulans*) bZIP-type transcription factors are involved in the response to various environmental stresses (Balázs *et al.*, 2010; Hagiwara *et al.*, 2008; Nguyen *et al.*, 2013; Qi *et al.*, 2013). 2013). The *ΔFvatfA* deletion mutant also showed increased sensitivity to agents that cause oxidative stress, such as hydrogen peroxide, *t*BOOH (generates peroxide stress) and menadione (causes superoxide stress), as well as Congo red, which causes cell wall stress.

The role of *FvatfA* in the production of fumonisins

The deletion of the *FvatfA* gene caused a drastic decrease in the production of fumonisins, the amount of FB1 and FB2 fell below the CE-MS detection limit. In parallel with this observation, we also measured the relative expression of three genes belonging to the *FUM* gene cluster, *fum1*, *fum8* and *fum21*, and found that the expression levels of *fum1* encoding a polyketide synthase, which is the key enzyme of fumonisin biosynthesis, and *fum8*, which encodes the α-oxoamine synthase enzyme, in the mutant were significantly reduced compared to the wild-type and complemented strains. No change was observed in the expression level of the *fum21* gene, which encodes a transcription factor containing a Zn(II)2Cys6 DNA binding site.

The role of *FvatfA* in bikaverin production

The *ΔFvatfA* deletion mutant produced about ten times more bikaverin after 5 days of incubation than the wild-type and complemented strains, but this difference did not increase after 7 and 9 days of incubation. The expression of the *bik1* gene, encoding the polyketide synthase enzyme involved in bikaverin biosynthesis, was examined 3 and 5 days after inoculation. In the case of the *ΔFvatfA* mutant, we did not observe an increase in gene expression levels compared to the wild-type, which suggests that the increased expression of the *bik1* gene is not responsible for the overproduction of bikaverin.

The role of *FvatfA* in the production of carotenoids

The deletion mutant produced only traces of carotenoids, while the wild-type and complemented strains produced these metabolites in normal amounts.

Among the genes involved in the biosynthesis of carotenoids, the relative expression of *carRA* and *carB* was significantly lower in the *ΔFvatfA* mutant than in the wild-type and complemented strains, while the expression of the *carT* gene was not affected by the deletion of the *FvatfA* gene.

Results of *in silico* promoter analysis

The ATF/CREB bZIP-type transcription factors can bind to the consensus sequence TGACGTCA (Sakamoto *et al.*, 2008; Hong *et al.*, 2013a), in case of genes involved in the production of the examined secondary metabolites we performed *in silico* promoter analysis to determine whether this sequence in the promoter regions of genes is present. We searched for the sequences of nine binding sites recognized by the ATF/CREB transcription factors occurring in various species included in the PROMO software. As expected, all genes that were down-regulated as a result of *FvatfA* gene deletion (*fum1*, *fum8*, *carRA* and *carB*) contained putative ATF/CREB binding sites in their promoters. In contrast, no potential binding sites were found in the promoter region of genes whose expression (*fum21*, *carT*, *bik1*) was not decreased in the mutant.

The *FvmnSOD* gene of *F. verticillioides*

The role of *FvmnSOD* in the stress sensitivity of *F. verticillioides*

Deletion of the *FvmnSOD* gene resulted in increased sensitivity to superoxide stress-inducing menadione. Despite the fact that there was no significant difference in the relative growth of the strains on agar plates supplemented with NaCl, KCl, and *t*BOOH, the morphology of *ΔFvmnSOD* colonies was slightly different from that of the wild-type.

To determine whether the *FvmnSOD* gene is involved in protection against PAF (*Penicillium chrysogenum* antifungal protein), the strains were inoculated onto Czapek-Dox agar supplemented with 100 or 200 μg/ml PAF. The deletion mutant was shown to be sensitive to PAF-induced apoptotic cell death.

The role of *FvmnSOD* in the production of fumonisins

Deletion of the *FvmnSOD* gene did not affect the production of FB1 and FB2, despite the fact that the *ΔFvmnSOD* strain showed increased sensitivity to superoxide stress-inducing menadione.

Role of *FvmnSOD* in *F. verticillioides* respiration and mitochondrial morphology

The volumetric ratio of mitochondria was determined using a laser confocal microscope in the apical second hyphal segments. As a result of the deletion of *FvmnSOD* gene, the percentage volumetric ratio of mitochondria increased in the regions examined. We found that the absence of the *FvmnSOD* gene has no effect on the level of intracellular relative superoxide radicals in the hyphae. We also found that the diameter of the hyphae in the deletion mutant was significantly smaller than in the wild-type.

Since the scene of respiration is in the mitochondria, we examined the levels of total, KCN-sensitive cytochrome c-dependent, SHAM-sensitive alternative oxidase, and KCN+SHAM-resistant residual respirations in mycelium samples from exponentially growing submerged cultures. The levels of total, KCN-sensitive and residual respirations were significantly higher in the deletion mutant. Deletion of the *FvmnSOD* gene had no effect on the alternative oxidase-dependent respiration of *F. verticillioides*. When the respiratory rates were divided by the volume occupied by the mitochondria, we found that there was no significant difference in the normalized respiratory rates between the wild-type and the *ΔFvmnSOD* deletion mutant.

Effects of *FvmnSOD* on invasive growth and sexual reproduction

The female fertility of *ΔFvmnSOD* was not lower than the fertility of the wild-type parent strain and the complemented strains, and we did not observe any differences in the invasion growth measured on tomatoes between the tested strains. All this suggests that *FvmnSOD* does not play a role in either sexual reproduction or invasive growth.

Dicussion and recommendations

Discussion of results related to the *FvatfA* gene

As we saw in the compilation above, the Atf1/AtfA transcription factors are essential in the formation of the response to various stresses, they also play a role in the production of secondary metabolites and in sexual reproduction.

According to our experiments, the vegetative growth of the *ΔFvatfA* mutant was retarded in the same way as was observed in the case of the previously described *atf1*/*atfA* orthologous gene deficiency mutants. The weak invasive growth of the mutant measured on tomato fruits suggests that the FvAtfA transcription factor plays a role in the virulence of *F. verticillioides*, as was also observed in other fungi (Nathues *et al.*, 2004; Guo *et al.*, 2010; Qi *et al.*, 2013; Nguyen *et al.*, 2013; Jiang *et al.*, 2015). Also in accordance with previous publications, inactivation of *FvatfA* in *F. verticillioides* caused disturbances in both asexual and sexual sporulation (Nathues *et al.*, 2004; Guo *et al.*, 2010; Nguyen *et al.*, 2013; Jiang *et al.*, 2015; Temme *et al.*, 2015; Qi *et al.*, 2013).

Deletion mutants of the *atf1*/*atfA* orthologs found in the literature were sensitive to various environmental stresses. *M. oryzae ΔMoatf1* and *F. graminearum ΔFgatf1* deletion mutant showed increased sensitivity to oxidative stress-inducing hydrogen peroxide (Guo *et al.*, 2010; Jiang *et al.*, 2015). The *Δbcatf1* mutant in *Botrytis cinerea* was found to be sensitive to Congo red and calcofluor white, which cause cell wall stress (Temme *et al.*, 2012). The *F. graminearum ΔFgatf1* mutant was also sensitive to NaCl-induced osmotic stress (Nguyen *et al.*, 2013; Jiang *et al.*, 2015), which was not observed in the case of the *F. verticillioides ΔFvatfA* mutant.

Several studies have highlighted that the production of secondary metabolites is related to the response of filamentous fungi to oxidative stress. The generally accepted theory is that antioxidant molecules form the first line of defense in response to ROS-induced oxidative stress, while secondary metabolites form the second line of defense (Roze *et al.*, 2011; Yin *et al.*, 2012; Hong *et al.*, 2013b). However, this statement may vary depending on the fungal species, the type of secondary metabolite, and environmental factors. In *F. graminearum*, the *ΔFgatf1* deletion mutant produced more deoxynivalenol (DON) after one and three days of incubation than the wild-type parent strain. Parallel to the increased DON production, the transcription of the *tri4*, *tri5*, *tri6* and *tri10* genes encoding the key enzymes of trichothecene biosynthesis increased in the mutant compared

to the wild strain. At the same time, the deletion mutant and the wild-type produced the same amount of zearalenone (ZEA), and the transcription level of the genes involved in ZEA biosynthesis (*zea1*, *zeb1*, *zeb2*) was almost the same (Nguyen *et al.*, 2013). In *B. cinerea*, the *ΔBcatf1* mutant produced significantly more botrydiol, botryenediol and botcinin A, and key genes involved in the biosynthesis of these phytotoxins were also up-regulated (Temme *et al.*, 2012).

Contradictory results were also obtained in our work when we examined the production of several metabolites in the same *ΔatfA* null mutant. The deletion of *FvatfA* was associated with the complete absence of fumonisin (FB1 and FB2) production and a drastic reduction in the production of carotenoids, while the mutant produced almost ten times more of another secondary metabolite, bikaverin, than the wild-type parent strain. The most important key genes of fumonisin and carotenoid biosynthesis were down-regulated in the mutant, but one of the key genes of bikeverin biosynthesis, *bik1*, did not show increased expression in the same mutant even in the case of tenfold overproduction of the metabolite. The fact that the initiation of the synthesis of certain secondary metabolic products is part of the response to stress is made clear by our results obtained during the *in silico* promoter analysis, ATF/CREB binding sites were found on the promoters of the *fum1*, *fum8*, and *carRA* and *carB* genes. This clearly proves that fumonisin and carotenoid biosynthesis genes are activated not only by their own, sequence-specific transcription factors (in fact, they are not sufficient by themselves), but also by global (general) transcription factors in induction, especially strong induction are also involved. FvAtfA is such a transcription factor! And how can we explain that the activity of the *bik1* gene did not increase in the *ΔFvatfA* mutant, while this mutant produced ten times as much bikaverin as the wild-type parent strain? We believe that due to the decline in the production of other metabolites (fumonisins, carotenoids), precursor molecules accumulated in excess amounts in the *ΔFvatfA* mutant cells, and these were channeled by the cell to the synthesis of bikaverin or other, currently unexamined metabolites. And the fact that the production of carotenoids dropped significantly in the *ΔFvatfA* mutant is also an important result, as it spectacularly supports the theory that the production of secondary metabolites is a response to oxidative stress. This result of ours provides strong (indirect) evidence that the Atf1/AtfA orthologous FvAtfA transcription factor involved in the formation of the oxidative stress response is essential for the production of antioxidant carotenoids.

There are elements that can be used in practice in this work, which originally started as exploratory research. (1) Our results contributed to a better

understanding of the complex regulation of mycotoxin production, and this can be used in plant breeding work aimed at reducing toxin production or in crop management technologies. (2) Non-mycotoxin-producing strains can be produced by not directly disrupting the genes controlling toxin biosynthesis; with this new type, we can edit so-called disarmed strains that can be used for (postharvest) biological control purposes. (3) Strains that overproduce industrially significant secondary metabolites (in our case, bikaverin) can be produced in such a way that by eliminating (or overproducing) transcription factors, the secondary metabolic product profile of the strain in this way is purposefully rearranged; this will obviously be a possible way for other fungi and other valuable metabolites as well. (4) Our results can also be used in the breeding of mycotoxin-free strains capable of producing protein mass (mycoprotein).

Discussion of results related to the *FvmnSOD* gene

The *ΔFvmnSOD* mutant of *F. verticillioides* showed increased sensitivity to superoxide stress-inducing menadione (MSB) and to apoptosis induced by *P. chrysogenum* antifungal protein (PAF), so the (putative) product of the gene, manganese-superoxide dismutase, is also present in this fungal species. Although the lack of *FvmnSOD* and the enzyme it encodes is not essential for the fungus, the lack had to be compensated somehow. In *F. verticillioides*, this occurred with an increase in the percentage volumetric ratio occupied by mitochondria; however, all this was coupled with higher energy consumption, and thanks to this, the fungus developed thinner hyphae. Under natural conditions, this obviously leads to a deterioration of competitiveness. The increased MSB and PAF sensitivity of the deficiency mutant also shows that this compensatory strategy did not work perfectly. According to our measurements, the relative superoxide ratio in the mutant did not change compared to the wild-type, and this is probably why the deletion of *FvmnSOD* had no effect on fumonisin production - in contrast to other metabolites in other fungi.

The production of SOD enzymes is considered a virulence factor in several plant pathogenic fungi. If the fungus can successfully handle the reactive oxygen forms produced as the first step in plant defense, this will facilitate its establishment and invasion. The *ΔFvmnSOD* mutant of *F. verticillioides*, on the other hand, did not prove to be weaker in colonization activity measured on tomatoes than the wild-type parent strain, which suggests that this species (which can live endophytically in corn tissues for months!) is a very efficient and complex ROS handler has a mechanism, of which the *mnSOD* enzyme is only one

and apparently not very significant element. This observation is also an important practical aspect of our *mnSOD* experiments, because it warns plant breeders that the ROS protection strategy of the pathogen to be overcome by breeding should be carefully analyzed when planning the selection strategy and selecting the selection method.

New scientific results

1. Using the toolbox of functional genomics, we verified that the *FvatfA* gene of *Fusarium verticillioides* (which encodes the Atf1/AtfA bZIP type transcription factor) plays a significant role in the oxidative and cell wall stress response.

2. The *FvatfA* gene is not essential for the fungus, but the vegetative growth of the Δ*FvatfA* mutant measured on synthetic media was weaker than that of the wild-type parent strain. The deletion mutant became female sterile, and its asexual spores were smaller than those of the wild-type, so the *FvatfA* gene affects both sexual and asexual reproduction.

3. The invasion growth of the Δ*FvatfA* mutant measured on tomato fruits was weaker than that of the wild-type, so the *FvatfA* gene can be considered a virulence factor.

4. The deletion of the *FvatfA* gene rearranged the secondary metabolite profile of the fungus: the deletion mutant did not produce fumonisins, carotenoid production was drastically reduced, and it produced significantly more bikaverin.

5. Using *in silico* promoter analysis, we proved that the promoters of key enzyme genes controlling fumonisin and carotenoid biosynthesis have ATF/CREB binding sites, which proves that FvAtfA as a general transcription factor supports the transcription of these genes (and through this, fumonisin and carotenoid production).

6. We also confirmed with functional genomic tools that the *FvmnSOD* gene (which, by derivation, encodes the enzyme manganese superoxide dismutase) plays a role in protection against oxidative stress and the preservation of mitochondrial integrity, but its absence does not cause or prevent either avirulence or female sterility fumonisin production.

7. Cells compensate for the lack of the *FvmnSOD* gene by increasing the percentage volume of mitochondria, but this compensation has limited efficiency.

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List of publications

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