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ORIGINAL PAPER

The bZIP-type transcription factors NapA and RsmA modulate the volumetric ratio and the relative superoxide ratio of mitochondria in *Aspergillus nidulans*

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Abstract

Basic leucine zipper (bZIP) transcription factors are crucial components of differentiation, cellular homeostasis and the environmental stress defense of eukaryotes. In this work, we further studied the consequence of gene deletion and overexpression of two bZIP transcription factors, NapA and RsmA, on superoxide production, mitochondrial morphology and hyphal diameter of *Aspergillus nidulans*. We have found that reactive oxygen species production was influenced by both gene deletion and overexpression of *napA* under *tert*-butylhydroperoxide (*t*BOOH) elicited oxidative stress. Furthermore, gene expression of *napA* negatively correlated with mitochondrial volumetric ratio as well as sterigmatocystin production of *A. nidulans*. High *rsmA* expression was accompanied with elevated relative superoxide ratio in the second hyphal compartment. A negative correlation between the expression of *rsmA* and catalase enzyme activity or mitochondrial volumetric ratio was also confirmed by statistical analysis. Hyphal diameter was independent on either *rsmA* and *napA* expression as well as 0.2 mM *t*BOOH treatment.

Keywords bZIP-type transcription factors · *t*BOOH stress · Superoxide level · Mitochondrial volumetric ratio · Hyphal diameter

Introduction

Basic domain leucine zipper (bZIP) transcription factor proteins are the members of the most ancient transcription factor families emerged from a single eukaryotic gene. They are centerpieces of a complex regulatory network, governing the maintenance and differentiation of cells as well as modulation of stress defense in eukaryotes (Jindrich and Degnan 2016). A subset of bZIPs called Yap (yeast activator protein) transcription factors and their orthologs have been thoroughly studied in fungi (Yin et al. 2013). The genome of the baker's yeast *Saccharomyces cerevisiae* accomodates eight Yaps (Fernandes et al. 1997; Rodrigues-Pousada et al. 2019), among which Yap1 is one of the key players orchestrating oxidative stress response (Rodrigues-Pousada et al. 2019; Yaakoub et al. 2022). Not surprisingly, Yap1 orthologs are wide-spread in other yeast species as well as in filamentous fungi (Nikolaou et al. 2009; Yaakoub et al. 2022), and are well-described in the genus *Aspergillus* (Asano et al. 2007; Reverberi et al. 2007, 2008, 2012; Qiao et al. 2008; Yin et al. 2013; Bok et al. 2014; Zheng et al. 2015; Mendoza-Martínez

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et al. 2017; Bákány et al. 2021). In addition to their pivotal role in the regulation of the oxidative stress defence system of the Aspergilli, they may also influence the biosynthesis of important secondary metabolites like the mycotoxins aflatoxins (AFs) and sterigmatocystin (ST) through modulating intracellular reactive oxygen species (ROS) levels (Reverberi et al. 2007, 2008, 2010; Yin et al. 2013; Bákány et al. 2021) and via regulating the induction of the gene clusters (Reverberi et al. 2008, 2010; Hong et al. 2013a, b). In the filamentous fungus model organism *A. nidulans*, deletion of *napA* (the *yap1* ortholog in this fungus) dramatically increased the oxidative stress sensitivity of the fungus in the presence of menadione sodium bisulfite, *tert*-butyl hydroperoxide (*t*BOOH) and H_2O_2, while deletion of *napA* facilitated and overexpression of *napA* hindered ST production (Yin et al. 2013; Bákány et al. 2021).

RsmA is also a bZIP-type transcription factor in the Aspergilli with greatest homology to Yap3 in *S. cerevisiae* (Yin et al. 2012). Interestingly, Yap3 may contribute to overcoming various types of environmental stress including hydroquinone and endoplasmic reticulum stress in baker's yeast (Rodrigues-Pousada et al. 2019). In *A. nidulans*, the overexpression of *rsmA* restores ST production in *ΔlaeA* and *ΔveA* strains (Shaaban et al. 2010; Yin et al. 2012), and also increases ST yields considerably (Shaaban et al. 2010; Yin et al. 2012, 2013; Bákány et al. 2021). LaeA and VeA are members of the Velvet complex, LaeA, which is a putative methyltransferase, is a global regulator of secondary metabolite production in the Aspergilli, while VeA is a light-dependent governor in the development and secondary metabolite production in several Aspergilli (Calvo 2008; Calvo and Cary 2015). Nevertheless, deletion of *rsmA* also seems to be beneficial for the biosynthesis of ST indicating a multiple regulation of ST production (Shaaban et al. 2010; Yin et al. 2013; Bákány et al. 2021). Importantly, the regulatory effect of RsmA on ST production of *A. nidulans* is independent of the intracellular reactive species (RS) concentration as quantified by 2',7'-dichlorofluorescein (DCF) formation (Halliwell and Gutteridge 2007), although RsmA slightly reduces intracellular specific catalase activity (Bákány et al. 2021).

In previous studies performed in our laboratory, we demonstrated that genetic manipulation of genes responsible for the maintenance of morphology and function of mitochondria in *A. nidulans* (Leiter et al. 2016) or *Fusarium verticillioides* (Szabó et al. 2020) may influence intracellular RS or superoxide (measured by dihydroethidium) concentrations, volumetric ratio of mitochondria within hyphae, relative superoxide ratio (in *F. verticillioides*), thickness of hyphae and, in the case of *A. nidulans*, ST production. Here we demonstrate for the first time that both NapA and RsmA negatively affect the volumetric ratio of mitochondria in *A. nidulans* and higher mitochondrial volumetric ratio is a pre-requisite of higher superoxide and ST productions.

Methods

Fungal strains, culture media and growth conditions

All strains tested in this study are listed in Table 1. Constructions of the *rsmA* and *napA* gene deletion and overexpression mutants have been described elsewhere (Yin et al. 2013; Bákány et al. 2021). All strains were maintained and sporulated at 37 °C on Barratt's nitrate minimal medium (NMM) supplemented with 0.05 mg l^{-1} pyridoxine (Barratt et al. 1965; Yin et al. 2013; Bákány et al. 2021). To determine superoxide production and examine the volumetric ratio of mitochondria, *A. nidulans* strains were cultured in Erlenmeyer flasks (500 ml) containing 100 ml NMM (pH 6.5) and also supplemented with 0.05 mg l^{-1} pyridoxine. In each experiment, culture media were inoculated with 10^6 conidia ml^{-1} and incubated at 37 °C and at 220 rpm shaking frequency. Oxidative stress was introduced by the addition of *t*BOOH (0.2 mM) to exponential growth phase (18 h) cultures as described before (Bákány et al. 2021). It is important to note that *t*BOOH is a lipid peroxidation stimulating agent (Halliwell and Gutteridge 2007), which also interferes with mitochondrial functions resulting in increased intracellular RS and superoxide productions (Fekete et al. 2007; Gazdag et al. 2014; Rogov et al. 2018).

Table 1 Strains used in this study

Name	Genotype	Reference
RDIT 9.32	Wild type	Tsitsigiannis et al. (2004)
RWY 2.12	*gpdA*(*p*)::*rsmA*::*A. fumigatus pyrG*	Yin et al. (2012)
RWY 8.5	Δ*rsmA*::*pyrG A. parasiticus*	Yin et al. (2013)
RWY 17.3	*A. fumigatus pyroA*::*gpdA*(*p*)::*napA*, *pyroA4*	Yin et al. (2013)
RWY 10.3	Δ*napA*::*pyroA A. fumigatus*	Yin et al. (2013)

All strains carry the wild type *veA* allele

Superoxide production and volumetric ratio of mitochondria

We measured superoxide anion radical ($O_2^{\cdot-}$) production, mitochondrial volumetric ratio and hyphal diameter in 23 h cultures [optimized in a previous study to measure physiological parameters after *t*BOOH treatment (Bákány et al. 2021)] of *A. nidulans* mycelia (i.e. 5 h after 0.2 mM *t*BOOH treatment as written in the previous section) using the following stains: dihydroethidium (reacts with $O_2^{\cdot-}$), MitoTracker Green (visualizes mitochondria) and Calcofluor White (visualizes chitin in cell walls) (Leiter et al. 2016; Szabó et al. 2020). Aliquots (1 ml each) of culture were transferred with pipette into 24 well culture plates and were stained sequentially with 100 nM MitoTracker Green (30 min), 10 nM dihydroethidium (20 min) and 5 nM Calcofluor White (5 min). Images were obtained using a LSM 880 (Zeiss, Oberkochen, Germany) laser scanning microscope (Leiter et al. 2016; Szabó et al. 2020). Three-channel confocal Z-stack images were captured where the green channel showed mitochondria stained by MitoTracker Green, the red channel visualized the oxidized forms of the superoxide indicator dihydroethidium, and the blue channel (Calcofluor White staining) showed the structure of the hypha and the boundaries for each segment. Volumetric ratio of mitochondria, relative superoxide ratio (superoxide volume [proportional to the formed ethidium signal] normalized to mitochondrial volume in %), as well as hyphal diameter in the second hyphal compartment from the apices were determined as before (Leiter et al. 2016; Szabó et al. 2020).

The microscopic images were analysed using custom-made software based on 2D stationary wavelet transform (SWT) denoising, as described in previous publications (Leiter et al. 2016; Szabó et al. 2020). Briefly, the region of interest (ROI), which is a single segment of the given hypha, was manually marked on each hyphae examined. The average intensity of the selected cell-free background on the picture was subtracted from intensity values. Mitochondria were identified by pixel intensity segmentation at the SWT W2 wavelet level. For each image, a noise-optimized threshold was used, above which the pixel was considered to belong to the mitochondria.

Statistical analysis of experimental data

The effects of *t*BOOH treatments and gene manipulations on the production of intracellular superoxide, mitochondrial volumetric ratio, relative superoxide ratio, and hyphal diameter were analysed by two-way ANOVA followed by Tukey's post-hoc test. The difference between the mean values as well as the interaction between the effects of gene manipulation and *t*BOOH treatment were regarded as significant if the adjusted *p*-value was less than 0.05. Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Pairwise correlations between the measured parameters were characterized by Pearson's correlation coefficients calculated using the mean values of the traits determined in the studied types of cultures. The following parameters were studied: superoxide production, mitochondrial volumetric ratio, hyphal diameter, relative superoxide ratio, as well as *napA* and *rsmA* expression (ΔC_P), reactive species (RS; includes all reactive oxygen and nitrogen species, which oxidize 2',7'-dichlorofluorescin to DCF; Halliwell and Gutteridge 2007), ST production, and specific catalase activity (Bákány et al. 2021).

Results

In this study we measured superoxide production, mitochondrial volumetric ratio and hyphal diameter in 23 h submerged cultures of *A. nidulans* mycelia using dihydroethidium, MitoTracker Green and Calcofluor White stains (Fig. 1), respectively.

Two-way ANOVA was performed to analyze the effect of genetic manipulation (reference strain *vs.* Δ *napA*, *OEnapA*, Δ *rsmA*, or *OErsmA* mutant) and *t*BOOH treatment (*t*BOOH treated vs. untreated) on superoxide ($O_2^{\cdot-}$) production (Fig. 2a). In the case of the Δ*napA* strain, the results indicated no significant main effect for *t*BOOH treatment, $F_{1,78} = 2.45$, $p = 0.1216$, partial $\eta^2 = 0.03$; significant main effect for *napA* gene deletion, $F_{1,78} = 8.19$, $p = 0.0054$, partial $\eta^2 = 0.10$; and a significant interaction between treatment and genetic manipulation, $F_{1,78} = 7.59$, $p = 0.0073$, partial $\eta^2 = 0.09$. In the case of the *OEnapA* strain no significant main effect for *t*BOOH treatment, $F_{1,83} = 3.45$, $p = 0.0667$, partial $\eta^2 = 0.04$; no significant main effect for *napA* overexpression, $F_{1,83} = 0.74$, $p = 0.3938$, partial $\eta^2 = 0.01$; and a significant interaction between treatment and genetic manipulation, $F_{1,83} = 13.21$, $p = 0.0005$, partial $\eta^2 = 0.14$, were observed. In the case of the Δ*rsmA* and *OErsmA* mutants, no significant main effects for *t*BOOH treatment ($F_{1,106} = 0.17$, $p = 0.6819$, partial $\eta^2 = 0.002$ and $F_{1,108} = 0.26$, $p = 0.6138$, partial $\eta^2 = 0.002$), no significant main effects for genetic manipulation ($F_{1,106} = 0.03$, $p = 0.8524$, partial $\eta^2 = 0.0003$ and $F_{1,108} = 1.57$, $p = 0.2127$, partial $\eta^2 = 0.01$), and no significant interactions between treatment and genetic manipulation ($F_{1,106} = 0.57$, $p = 0.4523$, partial $\eta^2 = 0.005$ and $F_{1,108} = 0.10$, $p = 0.7539$, partial $\eta^2 = 0.0009$) were calculated. According to the Tukey post-hoc tests, superoxide production in the *t*BOOH treated cultures of the Δ*napA* strain was significantly higher than in the *t*BOOH treated cultures of the reference strain ($p = 0.0009$) and in the untreated cultures of the Δ*napA* strain ($p = 0.0097$). Meanwhile, the superoxide production in the *t*BOOH treated cultures of the

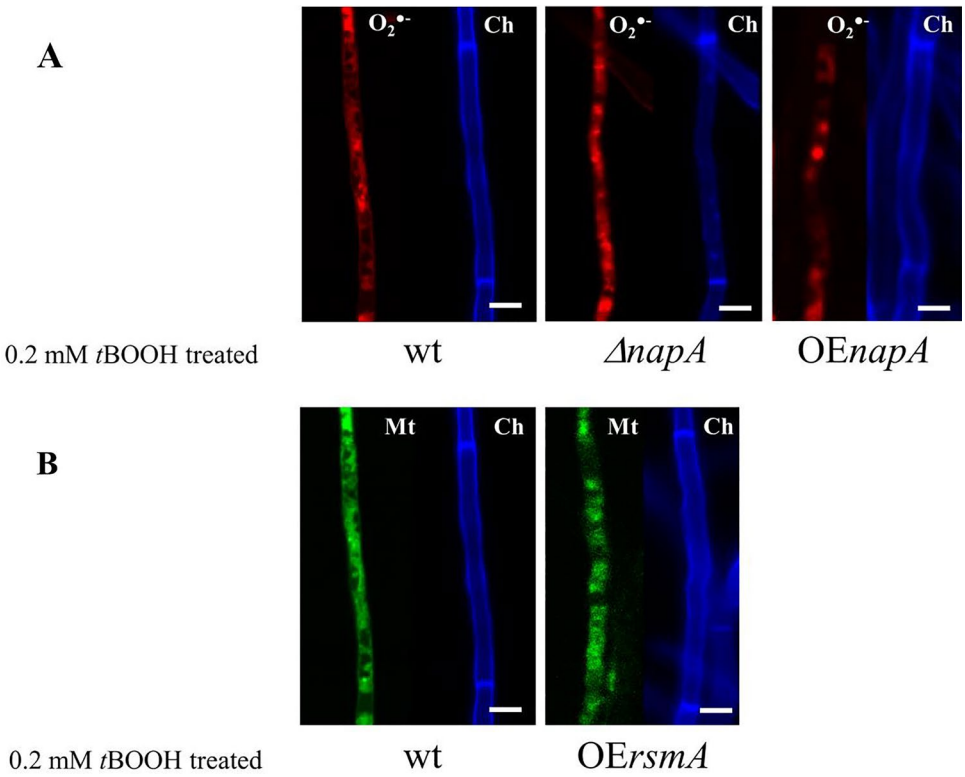

Fig. 1 Visualization and characterization of the superoxide production and mitochondria in *A. nidulans*. Three-channel confocal Z-stack images were taken where the green channel shows mitochondria (Mt) stained by MitoTracker Green, the red channel visualizes the superoxide ($O_2^{\bullet-}$) indicator dihydroethidium, while the blue channel (chitin staining by Calcofluor White, Ch) shows structure of the hypha and the boundaries for each segment. **a** Superoxide production of the wild type, Δ*napA* and OE*napA* mutants. **b** Comparing mitochondrial morphology in the second hyphal segment of the wild type and OE*rsmA* mutant. Representative images showing significantly different staining from wild type ($p < 0.05$). Scale bar: 10 µm

OEnapA strain was significantly smaller than in the *t*BOOH treated cultures of the reference strain ($p = 0.0084$) and in the untreated cultures of the Δ*napA* strain ($p = 0.0005$) (Fig. 2a).

The relative superoxide productions of the same cultures were also evaluated (Fig. 2b). In the case of the Δ*apA* and the *OErsmA* mutants, no significant main effects for *t*BOOH treatment ($F_{1,58} = 0.09$, $p = 0.7710$, partial $\eta^2 = 0.001$ and $F_{1,88} = 0.0007$, $p = 0.9792$, partial $\eta^2 = 0.00$), no significant main effect for gene manipulation ($F_{1,58} = 2.78$, $p = 0.1007$, partial $\eta^2 = 0.05$ and $F_{1,88} = 2.77$, $p = 0.0998$, partial $\eta^2 = 0.03$) and no significant interaction between treatment and genetic manipulation ($F_{1,58} = 2.90$, $p = 0.0940$, partial $\eta^2 = 0.05$ and $F_{1,88} = 2.15$, $p = 0.1462$, partial $\eta^2 = 0.02$) were observed. In the case of the *OEnapA* strain, the analyzes revealed significant main effects for *t*BOOH treatment ($F_{1,67} = 8.87$, $p = 0.0040$, partial $\eta^2 = 0.12$), no significant main effect for gene manipulation ($F_{1,67} = 0.20$, $p = 0.6553$, partial $\eta^2 = 0.003$) and no significant interaction between treatment and genetic manipulation ($F_{1,67} = 3.01$, $p = 0.0873$, partial $\eta^2 = 0.04$). With the Δ*rsmA* strain, no significant main effects for *t*BOOH treatment ($F_{1,86} = 1.64$, $p = 0.2036$, partial $\eta^2 = 0.02$), significant main effect for *rsmA* gene deletion ($F_{1,86} = 11.97$, $p = 0.0008$, partial $\eta^2 = 0.12$) and no significant interaction between treatment and genetic manipulation ($F_{1,86} = 0.43$, $p = 0.5158$, partial $\eta^2 = 0.005$) were calculated. The *t*BOOH treatment significantly decreased the relative superoxide production in the *OEnapA* strain ($p = 0.0109$), deletion of the *rsmA* gene also decreased the relative superoxide production both in the *t*BOOH treated ($p = 0.0062$) and untreated ($p = 0.0340$) cultures relative to the untreated cultures of the wild type strain according to the Tukey post-hoc test (Fig. 2b).

Considering the mitochondrial volumetric ratio (Fig. 2c), in the case of the gene deletion mutants (Δ *napA* and Δ *rsmA*), the two-way ANOVA revealed no significant main effects for *t*BOOH treatment ($F_{1,78} = 0.12$, $p = 0.7311$, partial $\eta^2 = 0.002$ and $F_{1,106} = 0.81$, $p = 0.3695$, partial $\eta^2 = 0.008$), no significant main effects for genetic manipulation ($F_{1,78} = 2.25$, $p = 0.1374$, partial $\eta^2 = 0.03$ and $F_{1,106} = 2.94$, $p = 0.0896$, partial $\eta^2 = 0.03$), and no significant interactions between treatment and genetic manipulation ($F_{1,78} = 1.04$, $p = 0.3115$, partial $\eta^2 = 0.01$ and $F_{1,106} = 1.24$, $p = 0.2687$, partial $\eta^2 = 0.01$). In the case of the over-expression mutants (*OEnapA* and *OErsmA*), no significant main effects for *t*BOOH treatment ($F_{1,83} = 0.07$, $p = 0.7817$, partial $\eta^2 = 0.0009$ and $F_{1,108} = 0.42$, $p = 0.5210$, partial $\eta^2 = 0.004$), significant main effects for over-expression ($F_{1,83} = 10.70$, $p = 0.0016$, partial $\eta^2 = 0.11$ and $F_{1,108} = 11.60$, $p = 0.0009$, partial $\eta^2 = 0.10$), but no significant interactions between treatment and genetic manipulation ($F_{1,83} = 0.30$, $p = 0.5837$, partial $\eta^2 = 0.004$ and $F_{1,108} = 0.33$, $p = 0.5659$, partial $\eta^2 = 0.003$) were observed. According to the Tukey post-hoc tests, the mitochondrial volumetric ratio in the *t*BOOH treated cultures of the *OEnapA* strain was significantly smaller than in the untreated reference strain ($p = 0.0428$) (Fig. 2b), while in the *t*BOOH treated cultures of the

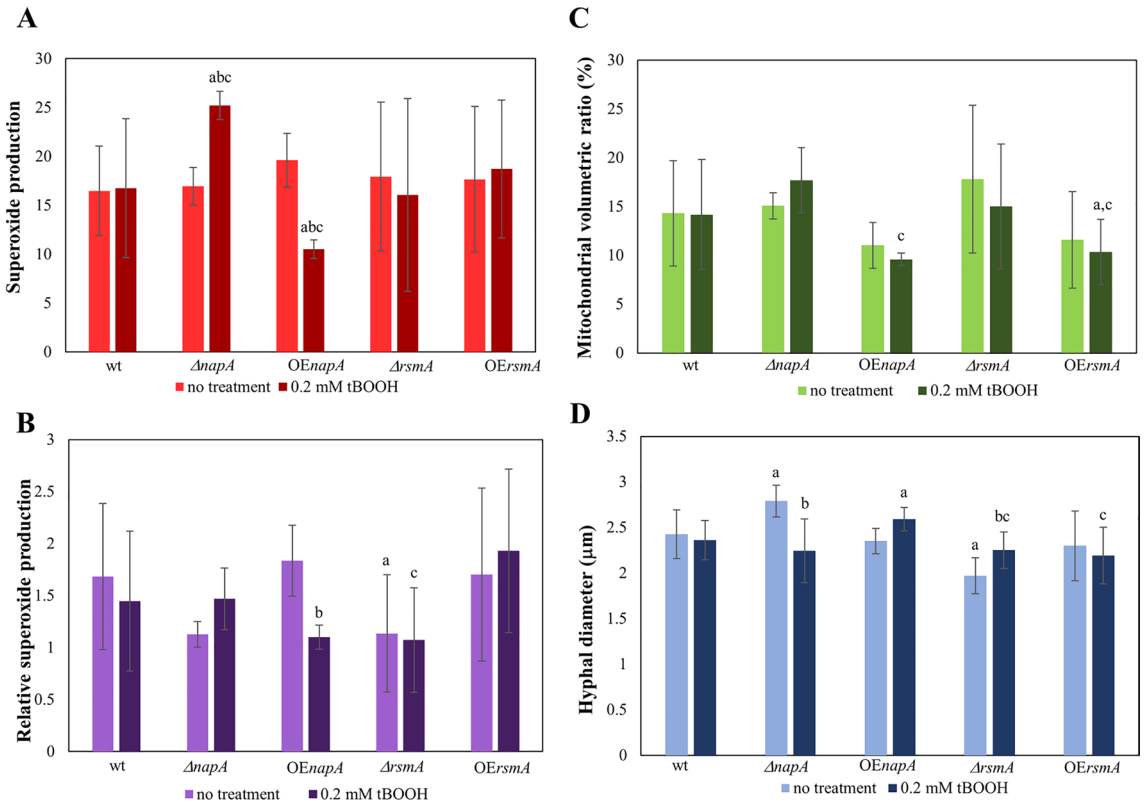

Fig. 2 **a** Superoxide production of the mutants in 23 h submerged cultures. The superoxide ratio was measured by dihydroethidium staining. **b** Relative superoxide (superoxide volume [proportional to the forming ethidium signal] normalized to mitochondrial volume in %) production of the wild type and mutant strains. **c** Comparison of the volumetric ratio (%) and size of mitochondria in the wild type and mutant strains. **d** Determination of the hyphal diameter of the wild type and mutant strains in the second hyphal segment. Data are shown as mean ± SD values; statistical analysis was performed by two-way ANOVA followed by Tukey post-hoc test. [a]Significant differences between the cultures of the mutant and the wild type strain treated in the same manner ($p = 0.05$). [b]Significant differences between *t*BOOH treated and untreated cultures of the same strain ($p = 0.05$). [c]Significant differences between the *t*BOOH treated cultures of mutant strain and the untreated cultures of the wild type strain ($p = 0.05$)

OErsmA mutant, it was smaller than in the *t*BOOH treated or untreated cultures of the wild type strain (Fig. 2c).

Regarding the hyphal diameter (Fig. 2d), two-way ANNOVA revealed significant main effect for *t*BOOH treatment and for genetic manipulation and significant interaction between them ($F_{1,55} = 10.25$, $p = 0.0023$, partial $\eta^2 = 0.16$; $F_{1,55} = 5.48$, $p = 0.0229$, partial $\eta^2 = 0.09$ and $F_{1,55} = 10.56$, $p = 0.0020$, partial $\eta^2 = 0.16$) in the case of the Δ*napA* strain. In the case of the *OEnapA* mutant, no significant main effects for *t*BOOH treatment and genetic manipulation ($F_{1,58} = 0.23$, $p = 0.6326$, partial $\eta^2 = 0.004$ and $F_{1,58} = 1.38$, $p = 0.2458$, partial $\eta^2 = 0.02$), but significant interaction between treatment and genetic manipulation ($F_{1,58} = 6.53$, $p = 0.0133$, partial $\eta^2 = 0.10$) were observed. With the Δ*rsmA* mutant, the results of the two-way ANNOVA indicated no significant main effect for *t*BOOH treatment ($F_{1,85} = 3.16$, $p = 0.0787$, partial $\eta^2 = 0.04$), significant main effect for *rsmA* gene deletion ($F_{1,85} = 32.75$, $p = 1.5\ 10^{-7}$, partial $\eta^2 = 0.28$), and significant interaction between treatment and genetic manipulation ($F_{1,85} = 13.42$, $p = 0.0004$, partial $\eta^2 = 0.14$). With the *OErsmA* strain, no significant main effects for *t*BOOH treatment ($F_{1,90} = 2.13$, $p = 0.1481$, partial $\eta^2 = 0.02$), significant main effect for *rsmA* over-expression ($F_{1,90} = 5.54$, $p = 0.0208$, partial $\eta^2 = 0.06$) and no significant interaction between them ($F_{1,90} = 0.11$, $p = 0.7463$, partial $\eta^2 = 0.001$) were calculated. According to the Tukey post-hoc tests, the hyphal diameter in the untreated cultures of the Δ*napA* strain was significantly higher than in the cultures of the untreated reference strain ($p = 0.0016$) or the *t*BOOH treated Δ*napA* strain (Fig. 2d). The hyphal diameter of the *OEnapA* strain after *t*BOOH treatment was also higher significantly ($p = 0.0487$) than that of the *t*BOOH treated wild type strain (Fig. 2d). The Δ*rsmA* strain, both in *t*BOOH treated and untreated cultures, had significantly thinner hyphae than the untreated wild type strain ($p = 0.0350$ and < 0.0000), and the *t*BOOH treatment significantly increased the hyphal diameter in this mutant ($p = 0.0003$) (Fig. 2d). In the case of the *OErsmA* strain the hyphal diameter was smaller in the

*t*BOOH treated cultures than that of the wild type strain in untreated cultures ($p = 0.0443$) (Fig. 2d).

Pairwise correlations also included a number of RS, ST, catalase production as well as *napA* and *rsmA* gene expression values (Bákány et al. 2021) in addition to the parameters generated in this study.

Importantly, a firm correlation based on Pearson's correlation coefficients was found between dihydroethidium staining-based superoxide and DCF staining-based RS levels, meanwhile catalase correlated negatively with relative superoxide ratios of mitochondria (Table 2).

In this study, we demonstrated for the first time that the volumetric ratio of mitochondria measured in *A. nidulans* hyphae positively correlated with both superoxide and ST productions meanwhile both *napA* and *rsmA* expression levels negatively correlated with mitochondrial volumetric ratio (Table 2). It is reasonable to assume that NapA controls negatively RS (including superoxide) production and, consequently, ST yields via decreasing mitochondrial volumetric ratio (Fig. 2, Table 2; Bákány et al. 2021). It is noteworthy that genetic manipulation of *napA* decreased significantly the relative superoxide ratio of mitochondria only in *t*BOOH-exposed OE*napA* cultures (Fig. 2b). Similarly, *ΔFvmnSOD* (*FvmnSOD* encodes a manganese superoxide dismutase.) deletion increased mitochondrial volumetric ratio without affecting relative superoxide ratio in unstressed cultures of the maize pathogenic fungus *F verticillioides* (Szabó et al. 2020).

In the case of RsmA, although this Yap-like bZIP-type transcription factor does not seem to transmit environmental stress signals to the sterigmatocystin biosynthetic gene cluster (Yin et al. 2013; Bákány et al. 2021), the negative correlation with mitochondrial volumetric ratio (Fig. 2c, Table 2) coincided with a negative effect on catalase activity (Table 2; Bákány et al. 2020) and a positive correlation with relative superoxide ratio of mitochondria (Fig. 2b, Table 2). Importantly, the latter link between RsmA functions and the redox homeostasis of *A. nidulans* cells remained hidden when superoxide or RS formations (Fig. 1, Table 2; Bákány et al. 2020) were compared to *rsmA* expression levels.

It is noteworthy that some minor hyphal diameter phenotypes were observed in this study as well. Deletion of *napA* in untreated cultures and overexpression of *napA* in *t*BOOH-treated cultures increased, while deletion of *rsmA* in untreated cultures decreased hyphal diameter (Fig. 2d). Treatment with *t*BOOH also decreased the hyphal diameter of the Δ*napA* strain but increased it in the Δ*napA* strain (Fig. 2d). In the case of all the three strains (i.e. Δ*napA*, *OEnapA*, and Δ*rsmA*) the interaction between the effect of gene manipulation and oxidative stress treatment was significant according to the two-way ANOVA (Fig. 2d). Nevertheless, Pearson's correlation coefficients coming from pairwise correlation studies did not confirm correlation of hyphal diameters with any physiological and gene expression parameters tested (Table 2).

Table 2 Correlation between the parameters of this study and including also *napA* and *rsmA* expression, DCF and catalase production as well as ST production (Bákány et al. 2021) measured in different cultures

	Superoxide production	Mitochondrial volumetric ratio	Hyphal diameter	Relative superoxide ratio	ST production	Catalase	DCF	ΔCp_{napA}	ΔCp_{rsmA}
Superoxide production	1	**0.5009**	-0.4164	0.4093	**0.6456**	-0.2406	**0.7426**	-0.4782	-0.0030
Mitochondrial volumetric ratio	**0.5009**	1	-0.3094	-0.4347	**0.5929**	0.2622	0.4808	**-0.7220**	**-0.7072**
Hyphal diameter	-0.4164	-0.3094	1	-0.21519	-0.0252	-0.3437	-0.1663	-0.3117	0.4698
Relative superoxide ratio	0.4093	-0.4346	-0.2152	1	-0.2272	**-0.6643**	0.0268	0.3859	**0.6243**
ST production	**0.6456**	**0.5929**	-0.0252	-0.2272	1	0.0167	**0.7575**	**-0.7964**	-0.2175
Catalase	-0.2406	0.2622	-0.3437	**-0.6643**	0.0167	1	-0.1956	0.1130	**-0.7807**
DCF	**0.7426**	0.4808	-0.1663	0.02685	**0.7575**	-0.1956	1	**-0.6200**	0.0211
ΔCp_{napA}	-0.4782	**-0.7220**	-0.3117	0.3859	**-0.7964**	0.1130	**-0.6200**	1	0.1965
ΔCp_{rsmA}	-0.0030	**-0.7072**	0.4698	**0.6243**	-0.2175	**-0.7807**	0.0211	0.1965	1

< -0.7 | < -0.6 | 0.5 < | 0.6 < | 0.7 <

Pearson's correlation coefficient was calculated using the mean values of the traits determined in the tBOOH-treated and untreated culture of the five strains ($n = 10$). Correlation coefficients higher than 0.5, or less than − 0.5 were regarded as notable positive or negative correlations, respectively

Discussion

The "oxidative stress theory of mycotoxin biosynthesis" (Reverberi et al. 2010) is an attractive hypothesis to explain why mycotoxigenic fungi produce a wide spectrum of secondary metabolites, whose physiological functions are often quite difficult to decipher in these microorganisms. This dispute may be resolved by demonstrating the antioxidant character of these compounds like it has been done for AFs more recently by Finotti et al. (2021). Another strong argument to support this theory arose from the involvement of Yap1 orthologous bZIP-type transcription factors in the regulation of AF or ST production in various *Aspergillus* spp. (Reverberi et al. 2007, 2008, 2010; Hong et al. 2013a, b; Yin et al. 2013; Bákány et al. 2021). Furthermore, NapA (the Yap1 ortholog transcription factor in *A. nidulans*) responds to elevations in intracellular RS levels and sets into operation adequate elements of the oxidative stress defence system to cope with this kind of environmental stress and it also modulates sterigmatocystin production (Yin et al. 2013; Bákány et al. 2021). Nevertheless, other transcriptional regulators like the bZIP-type, Yap3 orthologous transcriptional factor RsmA also influence ST production independently of the redox status of the cells (Yin et al. 2013; Bákány et al. 2021). To make this regulatory pattern even more complicated, a genetic and/or physical interaction between NapA and RsmA themselves seems to be likely in *A. nidulans* (Bákány et al. 2021) and RsmA itself can be oxidative stress responsive in other *Aspergillus* spp. (Sekonyela et al. 2013; Wang et al. 2020).

The fine-tuning of AF and ST productions by bZIP-type transcription factors is still largely unknown although their interactions with other regulatory elements of the oxidative stress defence system and mycotoxin biosynthetic pathways are likely (Reverberi et al. 2008, 2010; Hong et al. 2013a, b). The apparent lack of any correlation between *rsmA* expression and ST yields (Table 2) came from the fact that both *rsmA* deletion and overexpression increase ST production (Shaaban et al. 2010; Yin et al. 2013; Bákány et al. 2021).

Mitochondria are the major ROS (including $O_2^{\cdot -}$ and H_2O_2) producers in eukaryotic cells including yeasts (Pan 2011; Eleutherio et al. 2018; Larosa and Remacle 2018) and, hence, their redox homeostasis may have a crucial impact on oxidative damages and ageing of cells even in filamentous fungi including *Podospora anserina* (Scheckhuber et al. 2012; Hamann and Osiewacz 2022) and the Aspergilli (Grahl et al. 2012; Leiter et al. 2016; Garrido-Bazán et al. 2020). It is important to note that confocal microscopy with MitoTracker Green staining made possible to reveal novel functions for both NapA {modulation of the mitochondrial volumetric ratio to decrease intracellular reactive species (including superoxide) and ST production} and RsmA (modulation of the volumetric ratio of mitochondria and positive correlation with the relative superoxide production ratio of mitochondria) Yap-like bZIP transcription factors. Therefore, both modulation of the mitochondrial volumetric ratio and relative superoxide ratio seem to be valuable parameters when we aim at a deeper understanding of how Yap-like bZIP-type transcription factors modulate the oxidative stress response system of filamentous fungi. Oxidative stress initiating agents may disturb the redox homeostasis of fungal cells resulting in the elevation of intracellular ROS (including superoxide) levels and, subsequently, triggering apoptosis-like cell death (Leiter et al. 2005; Nassimi et al. 2019; Qu et al. 2019; Király et al. 2020).

Intact mitochondrial structure and function are important prerequisites of longevity and slow ageing in fungi. The mitochondrial quality control system is crucial in the elimination of ROS, damaged proteins and impaired mitochondria and, therefore, in the maintenance of mitochondrial stability. The size of mitochondria influences both cellular ageing and cell death processes, i.e. larger, filamentous like mitochondria increases the lifespan of either yeast or senescent filamentous fungus cells (in *Saccharomyces cerevisiae Δdnm1* and *P. anserina ΔPaDnm1* strains), while smaller mitochondrial structures are more vulnerable to apoptotic cell death as demonstrated by Scheckhuber et al. (2007, 2009). Both endogenous and exogenous ROS may impair mitochondrial function and integrity by decreasing the mitochondrial volumetric ratio as observed in this study in the OE*rsmA* mutant after *t*BOOH treatment. Nevertheless, this landscape is slightly more nuanced in *A. nidulans* where higher mitochondrial volume and number (e.g. observed in the *ΔdnmA* and *pimA*OE mutants) did not necessarily increased oxidative stress defence or reduced apoptotic cell death (Leiter et al. 2016). Importantly, the deletion of *FvmnSOD* gene in *F verticillioides* although significantly increased the volumetric ratio of mitochondria meanwhile it concomitantly decreased the oxidative stress (menadione) and PAF (*Penicillium crysogenum* antifungal protein, which induces apoptosis in sensitive fungi) sensitivities without affecting mycotoxin production (Szabó et al. 2020). Therefore, any possible correlation between mitochondrium size, function, stress tolerance, ageing and life-span should be considered with care in a species and gene specific manner.

Nevertheless, in this study we found a positive correlation between mitochondrial volumetric ratio and mycotoxin (sterigmatocystin, a precursor of aflatoxins) production as well as significant negative correlations with two bZIP-type transcription factors (NapA and RsmA) with pivotal roles in the orchestration of oxidative stress defence and mycotoxin production (Bákány et al. 2021).

Because these observations gave us the very first hints on the regulation of mitochondrial volumetric ratio in the Aspergilli our current research aims include a similar study in the aflatoxigenic species *Aspergillus flavus*. Future studies should also aim to elucidate similar regulatory functions for bZIP orthologs in other mycotoxigenic fungi including Fusaria. Hopefully, after completing a series of such studies we will be able to address even more complex questions, *e.g.* modulating mitochondrial functions (and, hence, energy production) via optimizing volumetric ratios through bZIP-dependent transcriptional changes. To make this goal more feasible, target genes of bZIPs taking part in the modulation of mitochondrial volumetric ratios should be identified.

Interestingly, significant decreases in hyphal diameters were also recorded in farnesol-exposed *Rhizoctonia solani* hyphae concomitantly with increasing intracellular $O_2^{\cdot-}$ production and progressing cell death similarly to our study where significant correlation has been found between the effects of *napA* and *rsmA* manipulations and *t*BOOH treatments on *A. nidulans* hyphal diameter (Nassimi et al. 2019).

In summary, our study confirmed that both NapA and RsmA negatively influence the mitochondrial volumetric ratio in *A. nidulans*. We also found correlation between mitochondrial volumetric ratio and superoxide as well as ST productions. Further research should aim shedding light on the gene expression pattern involved in mitochondrial morphology and function as well as ST biosynthesis modulated by NapA and RsmA.

Conclusion for future biology

bZIP type transcription factors orchestrate various cellular processes in eukaryotes including fungi. Among others they play a pivotal role in the regulation of oxidative stress defence, secondary metabolite production and the maintenance of mitochondrial integrity. Our study helps pave the way in the development of industrial strains with increased environmental stress tolerance, intact mitochondrial morphology and function and reduced mycotoxin production. These data can also be helpful for agricultural professionals to control and mitigate the mycotoxin contamination of food and feed crops.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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