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Characterization of *gfdB*, putatively encoding a glycerol 3-phosphate dehydrogenase in *Aspergillus nidulans*

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ABSTRACT

The genome of *Aspergillus nidulans* accommodates two glycerol 3-phosphate dehydrogenase genes, *gfdA* and *gfdB*. Previous studies confirmed that GfdA is involved in the osmotic stress defence of the fungus. In this work, the physiological role of GfdB was characterized *via* the construction and functional characterization of the gene deletion mutant *ΔgfdB*. Unexpectedly, *ΔgfdB* strains showed oxidative stress sensitivity in the presence of a series of well-known oxidants including *tert*-butyl-hydroperoxide (*t*BOOH), diamide as well as hydrogen peroxide. Moderate sensitivity of the mutant towards the cell wall stress inducing agent CongoRed was also observed. Hence, both Gfd isoenzymes contributed to the environmental stress defence of the fungus but their functions were stress-type-specific. Furthermore, the specific activities of certain antioxidant enzymes, like catalase and glutathione peroxidase, were lower in *ΔgfdB* hyphae than those recorded in the control strain. As a consequence, mycelia from *ΔgfdB* cultures accumulated reactive species at higher levels than the control. On the other hand, the specific glutathione reductase activity was higher in the mutant, most likely to compensate for the elevated intracellular oxidative species concentrations. Nevertheless, the efficient control of reactive species failed in *ΔgfdB* cultures, which resulted in reduced viability and, concomitantly, early onset of programmed cell death in mutant hyphae. Inactivation of *gfdB* brought about higher mannitol accumulation in mycelia meanwhile the erythritol production was not disturbed in unstressed cultures. After oxidative stress treatment with *t*BOOH, only mannitol was detected in both mutant and control mycelia and the accumulation of mannitol even intensified in the *ΔgfdB* strain.

1. Introduction

Fungi encounter abiotic and biotic environmental stresses frequently in their natural habitats and, therefore, they possess an effective stress response system to adapt to and survive under such harsh and randomly varying circumstances (Miskei et al., 2009; Gasch, 2007). Fungal stress response systems rely on both enzymatic and non-enzymatic elements to combat environmental challenges (Pócsi et al., 2004; Duran et al., 2010; Gasch and Werner-Washburne, 2002). For example, the production of glycerol - which is one of the main osmoregulatory solutes in fungal cells - plays an important role in the prevention of hyperosmotic stress (Fillinger et al., 2001; Hagiwara et al., 2016; Saito and Posas, 2012). Glycerol can be produced in two steps including the enzyme glycerol 3-phosphate dehydrogenase (G3PDH), which catalyses the conversion of dihydroxyacetone phosphate (DHAP) into glycerol 3-phosphate (G3P), and G3P is subsequently converted into glycerol by a phosphatase (Fillinger et al., 2001).

In the baker's yeast *Saccharomyces cerevisiae*, two homologous genes, *GPD1* and *GPD2*, have been identified, which encode glycerol 3-phosphate dehydrogenases with different physiological functions. Meanwhile *GPD1* is involved in osmoadaptation *GPD2* is important in the maintenance of anaerobic growth of yeast cells (Ansell et al., 1997). In the fission yeast *Schizosaccharomyces pombe*, also two isoforms of glycerol 3-phosphate dehydrogenase, GPD1 and GPD2, were identified (Ohmiya et al., 1995). As expected, the

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gpd1Δ mutant showed hyperosmotic sensitivity, supporting the view that the *GPD1* gene is involved in osmoregulation in fission yeast (Ohmiya et al., 1995). Although *GPD2* likely had a different role rather than osmoadaptation but the construction of *gpd2::ura4+* haploid mutant failed in this yeast (Ohmiya et al., 1995).

In the filamentous fungus model organism *Aspergillus nidulans*, there are also two homologous genes, *gfdA* and *gfdB*, present in the genome (de Vries et al., 2017). The physiological function of *gfdA* was investigated in an earlier study by Fillinger et al. (2001), and the deletion of *gfdA* resulted in reduced growth with abnormal hyphal morphology on various carbon sources except with glycerol. Cell wall stress initiating agents like calcofluor white as well as sodium dodecyl sulfate aggravated the growth defect of *ΔgfdA* especially in low osmolarity environments (Fillinger et al., 2001).

In the human pathogenic fungus *Aspergillus fumigatus*, *gfdA* but not *gfdB* was essential for normal surface growth in glucose containing medium (Zhang et al., 2018). In the absence of *gfdA*, normal colony growth could be restored in hyperosmotic conditions suggesting that *gfdA* has a different function than its homologues gene *gpd1* in *S. cerevisiae* (Zhang et al., 2018).

In this study, we investigated the physiological functions of *A. nidulans gfdB* through the construction of gene deletion mutant strains and also compared their roles to those described before for GfdA (Fillinger et al., 2001). Although both enzymes are important in the environmental stress defence of *A. nidulans*, their functions seem to be complementary rather than overlapping. Meanwhile GfdA seems to be a key element of the osmotic stress defence system of the fungus GfdB finds its functions mostly in oxidative {*tert*-butyl-hydroperoxide (*t*BOOH), diamide, H_2O_2} and cell wall integrity (CongoRed) stress defences. Disturbances recorded in the reactive species (RS) productions and in specific antioxidant enzyme (catalase, glutathione peroxidase, superoxidase dismutase, glutathione reductase) activities of a *ΔgfdB* gene deletion strain are also presented and discussed here. Importantly, mannitol was the major alditol stress defence molecule in both stressed (*t*BOOH) and unstressed cultures of the same *ΔgfdB* strain.

2. Materials and methods

2.1. Strains, culture media, and production of conidia

The following *A. nidulans* strains were used in our study: rJMP1.59 (*pyrG89*; *pyroA4*; *veA*$^+$), rRAW16 (*pyrG89*; *yA2*; *veA*$^+$), THS30.3 (*pyrG89*, *AfupyrG*$^+$; *pyroA*$^+$; ve*A*$^+$) and *ΔgfdB* (*pyrG89*; *ΔgfdB*::*AfupyrG*$^+$; *pyroA+*; *veA*$^+$). Please note that four *ΔgfdB* strains, KA1-4, were selected for stress sensitivity phenotype screening, and one of them, strain KA1, was used in later physiological studies. All strains were maintained on Barratt's nitrate minimal medium (NMM) (Barratt et al., 1965), and NMM agar plates were incubated at 37 °C for 6 d (Balázs et al., 2010). Conidia harvested from these 6-day-old plates were used in all further experiments.

2.2. Construction of ΔgfdB gene deletion strains

The *gfdB* {encoding a putative glycerol 3-phosphate dehydrogenase, locus ID: AN6792; for more information consult the databases AspGD (http://www.aspergillusgenome.org/cgi-bin/locus.pl?locus=GfdB&organism=A_nidulans_FGSC_A4) and Fungal Stress Response Database (http://internal.med.unideb.hu/fsrd2/?p=consortium); Karányi et al. (2013), de Vries et al. (2017)} deletion mutants were constructed by the Double-Joint PCR method of Yu et al. (2004) and Leiter et al. (2016) with primers listed in Supplementary Table S1. The amplified deletion cassette was used to transform rJMP1.59 strain using the Vinoflow FCE lysing enzyme (Szewczyk et al., 2006). Single copy transformants were selected after Southern blot analysis and crossed with rRAW16 to get prototrophic strains (Leiter et al., 2016). All progenies of the independent crosses proved to be single-copy deleted mutants by Southern analyses.

2.3. Stress sensitivity studies

To study the stress sensitivity of the *ΔgfdB* mutant strains, the agar plate assays of Balázs et al. (2010) were adapted. The following stress generating agents were tested: oxidative stress: 6.0 mM hydrogen peroxide (causing peroxide stress), 2.0 mM diamide (eliciting GSH/GSSG redox imbalance), 0.8 mM *tert*-butyl hydroperoxide (*t*BOOH, triggering lipid peroxidation), 0.08 mM menadione sodium bisulfite (MSB, increasing intracellular superoxide level) (Pócsi et al., 2005; Emri et al., 1997); hyperosmotic stress: 2.0 M sorbitol, 1.5 M NaCl and 1.5 M KCl; cell wall integrity stress: 54 μM CongoRed (an agent known to alter cell wall polymer composition) (Kovács et al., 2013); heavy metal stress: 0.3 mM cadmium chloride. Plates were point-inoculated with 5 μl freshly made conidia suspension (2×10^7 conidia ml^{-1}) and were incubated at 37 °C for 5 d (Balázs et al., 2010). Diameters of the colonies were measured and used for the characterization of the stress sensitivities of the strains. In all stress sensitivity studies, the isogenic prototrophized THS30.3 (*pyrG89*, *AfupyrG*$^+$; *pyroA*$^+$; ve*A*$^+$) strain was used as the control strain.

2.4. Determination of physiological parameters in submerged cultures of A. nidulans

A. nidulans strains (THS30.3 control, *ΔgfdB*) were pre-grown in Erlenmeyer flasks (500 ml) containing 100 ml aliquots of NMM (pH 6.5). Culture media were inoculated with 10^8 spores and incubated at 37 °C and at 220 rpm shaking frequency. Oxidative stress was induced by the addition of 0.4 mM *t*BOOH to late exponential growth phase (20 h) cultures, and samples were taken after 10 and 24 h stress exposures for the determination of selected physiological parameters (Yin et al., 2013).

The intracellular reactive species (RS) levels were characterized by the formation of 2′,7′-dichlorofluorescein (DCF) from 2′,7′-dichlorofluorescin diacetate (Halliwell and Gutteridge, 2007). RS includes all Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), which oxidize 2′,7′-dichlorofluorescin to DCF (Halliwell and Gutteridge, 2007). At the incubation times tested, 10 μM 2′,7′-dichlorofluorescin diacetate was added to 20 ml aliquots of the cultures, and after incubating further for 1 h in 100 ml culture flasks, the mycelia were harvested by centrifugation. The production of DCF was determined spectrofluorimetrically (Emri et al., 1997, 1999).

Changes in the specific activities of certain antioxidative enzymes were also followed in separate experiments. Mycelium harvested by filtration was washed with distilled water and resuspended in ice-cold 0.1 M potassium phosphate buffer (pH 7.5). In these cases, cell-free extracts were prepared by disruption with 0.5 mm glass beads (5000 rpm, 30 s) and centrifugation (Emri et al., 1997). Specific catalase (Roggenkamp et al., 1974), glutathione peroxidase (GPx; Chiu et al., 1976), glutathione reductase (GR; Pinto et al., 1984) and superoxide dismutase (SOD; Oberley and Spitz, 1984) activities were measured according to the literature shown in parentheses.

Dry cell mass (DCM) of the samples was determined as described previously (Emri et al., 2004a, b), and protein contents of the cell-free extracts were measured by a modification of the Lowry method (Peterson, 1983).

2.5. Determination of mycotoxin production

To determine sterigmatocystin production, mycelia from 24 h stress exposed cultures were filtered and washed. After lyophilisation, sterigmatocystin was extracted with 70 % (v/v) acetone from 20 mg quantities of the freeze-dried mycelial powder. The sterigmatocystin content of the solutions was quantified on silica gel according to Klich et al. (2001).

2.6. Detection of the viability of the cultures

A modification of the method of Lee et al. (1999) was used to measure the specific MTT (methylthiazoletetrazolium) reducing activity of the cells, which is a widely used marker of cell viability. Mycelia from 0.5 ml culture aliquots of cultures were transferred into test tubes containing 0.5 ml fresh media supplemented with 10 mg ml^{-1} MTT. The mixtures were incubated for 4 h at 37 °C, then 0.3 ml of 200 g l^{-1} SDS in 20 mM HCl solution were added and the incubation continued for another 20 h. After centrifugation (10 000 g, 5 min), the MTT-formazan content of the supernatant was measured spectrophotometrically at 550 nm (A_{550}).

2.7. Measurement of mitochondrial membrane potential

Staining of apoptotic cells was assayed by JC-1 Mitochondrial Membrane Potential Detection Kit (Biotium) according to the manufacturer's instructions using JC-1 fluorescent cationic dye (5,5′,6,6-tetrachloro-1,1′,3,3-tetraethyl-benzimidazolocarbocyanin iodide). Samples were exposed to excitation at 485 nm and at 550 nm using a spectrofluorometer. The ratio of relative fluorescence intensity (RFU) was calculated by comparing the ratio of green and red fluorescence maxima: $RFU_{535\ nm}/RFU_{600\ nm} \times 100$ (Ádám et al., 2008).

2.8. Determination of the alditol content of mycelia

Alditols in *A. nidulans* mycelia were measured by HPLC. 20 mg lyophilized sample was dissolved in 1000 µl water. After centrifugation and filtration on 0.2 µm syringe filter 10 µl sample was injected to the column. HPLC conditions were as follows: column: Aminex HPX-87H (5 µm, 4.6*150 mm), eluent: 5 mM H_2SO_4 with flow rate of 1 ml min^{-1}, detection: diode array detector (DAD), 191 nm (Huber and Bonn, 1995). Quantitative measurements based on peak areas were made after calibration to the appropriate standards.

2.9. Preparation of mycelium and mycelial extracts for NMR measurements

A modification of the method of Witteveen et al. (1990) was used to adjust NMR-based metabolomics on the *ΔgfdB* mutant strain to determine the changes in the concentrations of some glycolytic and glycerol biosynthesis elements. The lyophilized mycelium was extracted with 0.1 M 35 % (w/w) deuterium chloride (DCl), and this suspension was cooled on ice and vortexed thoroughly. Then the extract was centrifuged at 4 °C for 10 min at 13,000 rpm. The supernatant was neutralized with K_2CO_3, then the precipitate was again removed by centrifugation and supernatant was used for NMR measurements. The alditols were identified by ^{1}H and ^{13}C NMR spectroscopy, in agreement with literature data (Witteveen et al., 1990). The NMR spectra were recorded at 298 K on Bruker Avance II and Avance NEO spectrometers operating at 500 and 700 MHz ^{1}H frequency, respectively.

2.10. Statistical analysis of experimental data

All experiments were performed in three independent sets, and mean ± SD values were calculated and are presented. Statistical significances were calculated using Student's *t*-test, and *p*-values less than 5 % were considered as statistically significant.

3. Results

3.1. Stress sensitivity phenotypes of the ΔgfdB mutants

ΔgfdB gene deletion strains showed reduced growth (5.1 ± 0.2 cm *vs.* 6.3 ± 0.2 cm colony diameters, when the KA1 mutant was compared to THS30.3 control) on minimal medium at 37 °C, and increased sensitivity to oxidative stress inducing agents like diamide, *t*BOOH and hydrogen-peroxide. In addition, we also demonstrated the moderately increased cell wall integrity stress (Congo Red) sensitivity of the *ΔgfdB* strain (1.8 ± 0.1 cm *vs.* 3.0 ± 0.2 cm colony diameters with 47.6 ± 3.4 % vs 35.1 ± 2.7 % growths compared to the untreated samples, using KA1 mutant and THS30.3 control strains; n = 3, p<5 %). No significant differences were observed between the growth of the control and the *ΔgfdB* gene deletion strains in the presence of 2 M sorbitol and 0.3 mM $CdCl_2$ (Fig. 1, Fig. S1). Since all the *ΔgfdB* progenies (KA1-4) showed the same stress sensitivity phenotypes (Fig. S1) *ΔgfdB* stands for the KA1 mutant in the forthcoming chapters of this publication, unless otherwise indicated.

Stress sensitivities of the strains were also tested in agar surface cultures grown on various carbon sources including 1 % glucose, fructose, glycerol and ethanol. Importantly, no increased oxidative stress (0.8 mM *t*BOOH) sensitivity was observed with the *ΔgfdB* strain in the presence of 1 % fructose as a sole carbon source, and 1 % glycerol also alleviated the growth inhibitory effect of *t*BOOH (Fig. S2). It is important to mention that there was no difference in colony diameters of the THS30.3 control strain cultivated on either glucose or glycerol carbon sources in the presence of *t*BOOH (Fig. S2).

Osmotic stress sensitivities of the strains were also determined on agar surface cultures grown on various carbon sources (Figs. S3–4). The differences in colony diameters between the control and *ΔgfdB* strains remained but were less pronounced on fructose, glycerol and ethanol carbon sources (Figs. S3–4). The addition of 1 M NaCl ameliorated the growth defect of the *ΔgfdB* strain slightly in the presence of 1 % glucose but this result was not statistically significant (Fig. S3). Contrarily, the addition of 2 M sorbitol to the culture medium did not increase the growth of the *ΔgfdB* at all on any of the carbon sources tested (Fig. S4) (Fillinger et al., 2001).

3.2. Specific RS and specific antioxidant enzyme productions

Significant increases were observed in specific reactive species productions in the *ΔgfdB* strain as compared to the THS30.3 control strain, both in the absence [1.0 ± 0.1 *vs.* 0.25 ± 0.03 (mmol DCF) (kg DCM)$^{-1}$] and in the presence of 0.4 mM *t*BOOH [8.0 ± 0.9 *vs.* 3.2 ± 0.7 (mmol DCF) (kg DCM)$^{-1}$] (Table 1), meanwhile no differences were observed in the biomass productions between the control and mutant strains after *t*BOOH treatments (Table 1.).

The specific activities of some antioxidant enzymes, including GPx, GR, catalase and SOD were measured to study the oxidative stress defence system of the mutant. Without any *t*BOOH treatment, the deletion of the *gfdB* gene increased the specific GR activity, while the GPx and the catalase activities were lower and SOD levels of the deletion strain was slightly but not significantly higher than those found in the THS30.3 control strain. However, in the

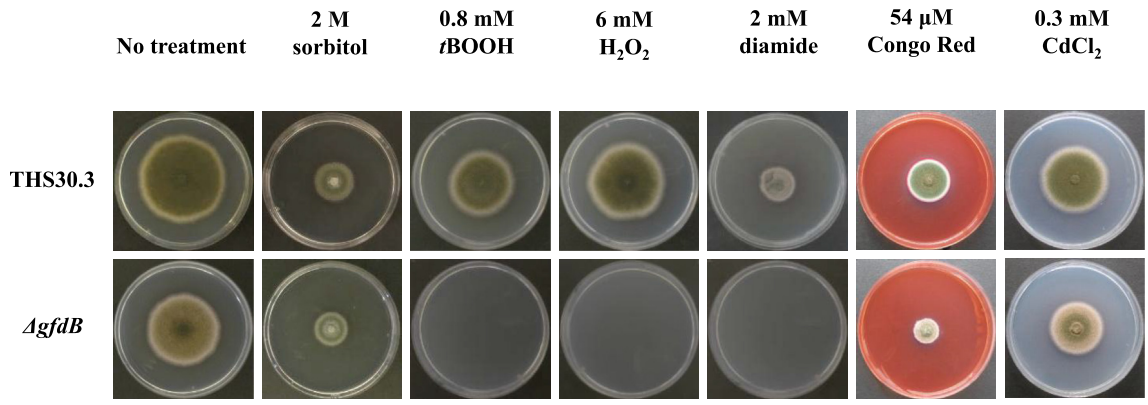

Fig. 1. Stress sensitivities of the THS30.3 (control) and the *ΔgfdB* gene deletion mutant strains exposed to various types of stress. Stress sensitivities observed in surface cultures on NMM agar plates are shown. NMM agar plates were incubated at 37 °C for 5 d.

Table 1
Biomass, sterigmatocystin, RS and antioxidant enzyme production in *Aspergillus nidulans* wild type (THS30.3) and *ΔgfdB* mutant strains.

Strains and treatments[a]	Biomass[b] (DCM) production [g (l culture)$^{-1}$]			Sterigmatocystin production [mg (g DCM)$^{-1}$]	RS[c] production [(mmol DCF) (kg DCM)$^{-1}$]	Glutathione reductase (GR) [mkat (kg protein)$^{-1}$]	Glutathione peroxidase (GPx) [mkat (kg protein)$^{-1}$]	Catalase [kat (kg protein)$^{-1}$]	Superoxide dismutase (SOD) [mkat (kg protein)$^{-1}$]	Alditol content [µmol (g DCM)$^{-1}$]
	0 h	10 h	24 h	24 h	10 h	10 h	10 h	10 h	10 h	24 h
THS30.3	1.6 ± 0.2	3.6 ± 0.03	4.3 ± 0.1	0.33 ± 0.05	0.25 ± 0.03	8.7 ± 1.8	16.4 ± 4.3	1.3 ± 0.4	82 ± 9	251 ± 91[d] 429 ± 115[e]
THS30.3 + *t*BOOH	1.6 ± 0.3	1.6 ± 0.5##	4.9 ± 0.4	0.07 ± 0.01##	3.2 ± 0.7##	8.1 ± 2.4	12.5 ± 3.4	2.8 ± 0.3	174 ± 66##	434 ± 16[e]
ΔgfdB	1.74 ± 0.09	3.5 ± 0.5	4.9 ± 0.5	0.57 ± 0.04**	1.0 ± 0.1***	18.2 ± 3.1***	12.3 ± 0.5*	0.5 ± 0.1***	96 ± 42	273 ± 12[d] 632 ± 50[e,*]
ΔgfdB + *t*BOOH	1.8 ± 0.1	1.8 ± 0.3##	4.9 ± 0.2	0.14 ± 0.08##	8.0 ± 0.9**###	13.9 ± 1.5***#	8.1 ± 1.8*###	1.2 ± 0.2***	62 ± 12**	812 ± 99[e,**#]

[a] Specific RS productions as well as specific enzyme activity values are presented as mean ± SD values calculated from three independent experiments. Significant differences between control and mutant cultures (*, p<5 %, **, p<1 % and ***, p<0.1 %) as well as between untreated and treated (0.4 mM *t*BOOH) cultures (#, p<5 %, ##, p<1 % and ###, p<0.1 %) are also presented, and *p* values were calculated using the Student's t-test.
[b] DCM, dry cell mass.
[c] RS includes all intracellular oxidants which react to give rise to 2′,7′-dichlorofluorescin diacetate (Halliwell and Gutteridge, 2007).
[d] erythritol.
[e] mannitol.

presence of *t*BOOH, SOD activities of the *ΔgfdB* strain were approximately three times lower than those recorded for the control (Table 1).

3.3. Sterigmatocystin production

Sterigmatocystin production was significantly higher in the mutant strain when compared to the control under unstressed culture conditions (Table 1). In the presence of *t*BOOH, sterigmatocystin production decreased in both strains but remained twice higher in the *ΔgfdB* mutant (Table 1).

3.4. Alditol content of mycelia

Concerning alditol productions in stressed and unstressed mycelia, meanwhile erythritol production remained unaltered, the mannitol concentration was significantly higher in the *ΔgfdB* strain under unstressed culture conditions as measured by HPLC (Table 1). After *t*BOOH (0.4 mM) treatments, erythritol production was hampered and inactivation of *gfdB* resulted in a significantly elevated mannitol production in comparison to the THS30.3 control as well as to the untreated *ΔgfdB* cultures (Table 1). It is worth noting that oxidative stress did not affect the mannitol production of the control strain (Table 1). Surprisingly, glycerol was not detected in submerged cultures of either the control or the mutant strains. Importantly, NMR measurements confirmed fully the HPLC analysis data concerning the alditol contents of mycelia (Fig. S5).

3.5. Viability and apoptotic cell death of the cultures

To compare the viabilities of the control and *ΔgfdB* strains, the MTT reducing capabilities of submerged cultures were measured. The viability of the control and *ΔgfdB* mutant decreased substantially after 52, 76 and 100 h total incubation times even in the presence or absence of *t*BOOH (Fig. 2) but the loss of MTT reducing activity was more pronounced in the *ΔgfdB* mutant. The loss of cell viability was accompanied with the increase in apoptotic cell death rate measured by the JC-1 fluorescent dye (Fig. 3.) (Ádám et al., 2008). Deletion of the *gfdB* gene resulted in about a three-fold increase in apoptosis rate after 100 h incubation without any *t*BOOH treatment (Fig. 3), while addition of this oxidative stress generating agent to the cultures triggered programmed cell death much earlier, after 52 h incubation time. The apoptotic cell death rate of the mutant was also significantly higher than those found with the THS30.3 control strain (Fig. 3).

4. Discussion

Glycerol is one of the most considerable compatible solutes in fungi including *A. nidulans* (Fillinger et al., 2001; Beever and Laracy, 1986). Glycerol can be synthetized in two consecutive steps in this

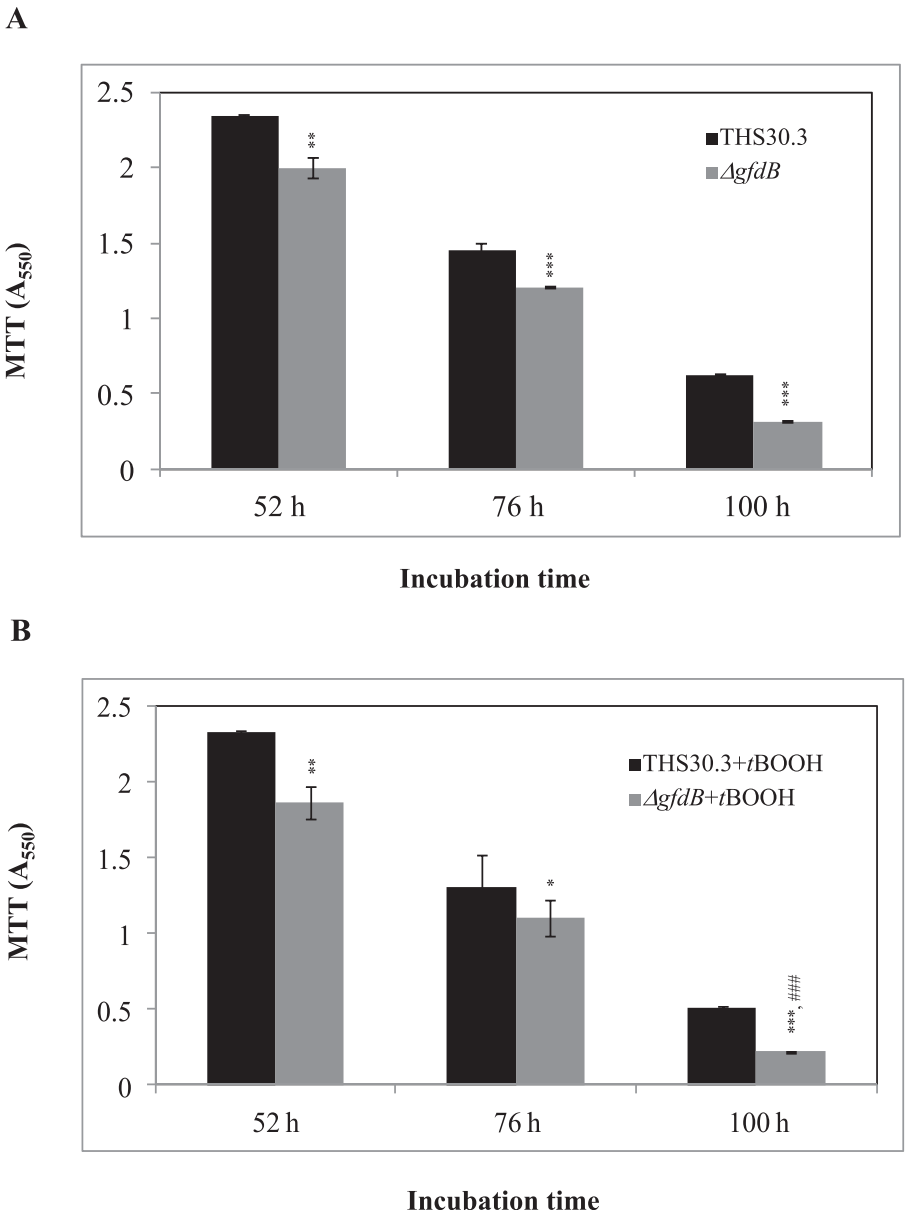

Fig. 2. Changes in the MTT reducing activities of THS30.3 (control) and *ΔgfdB* cultures. The specific MTT reducing activities of the cells was measured spectrophotometrically at 550 nm (A_{550}), which are presented as mean ± SD values calculated from three independent experiments. *t*BOOH (0.4 mM) was added to the cultures at 20 h incubation, and incubation times indicate the overall times lasted since inoculation with conidia. Significant differences between control and mutant cultures (*, p<5 %, **, p<1 % and ***, p<0.1 %) as well as between untreated and treated (0.4 mM *t*BOOH) cultures (#, p<5 %, ##, p<1 % and ###, p<0.1 %) are also indicated.

fungus by the enzymes glycerol 3-phosphate dehydrogenase and a putative phosphatase (Fillinger et al., 2001). Putative glycerol 3-phosphate dehydrogenases are encoded by two paralogous genes, *gfdA* and *gfdB*, accommodated by the genome of *A. nidulans* (de Vries et al., 2017). The physiological functions of *gfdA* have been investigated and discussed by Fillinger et al. (2001) and, in this study, we demonstrated that the physiological roles attributable to *gfdB* are remarkably different from those of *gfdA* in *A. nidulans*.

Inactivation of *gfdB* resulted in higher oxidative stress sensitivity compared to the control strain but, in contrast to *gfdA*, no hyperosmotic phenotype was observed, which indicated that *gfdB* was not responsible for the osmotic stability of this fungus. On the other hand, the oxidative stress sensitivity phenotypes of the *ΔgfdB* strain and the higher RS levels manifested in the *ΔgfdB* mutant even under unstressed conditions suggested that *gfdB* should be an important element of the oxidative stress defence of the fungus. Interestingly, the oxidative stress defence system of the *ΔgfdB* mutant seemed to need some fortifications, *e.g.* through increasing the specific GR activities to combat the increased RS levels. Nevertheless, since the specific GPx and catalase activities were significantly lower in the mutant strain the induction of specific GR activity was not satisfactory to cure the redox imbalance of the cells caused by the loss of *gfdB*. It is important to note that in *A. fumigatus* the inactivation of *gfdB* did not result in decreased growth in glucose-containing medium and the *gfdA* gene could not be substituted functionally by *gfdB* even by overexpressing *gfdB* in the *ΔgfdA* mutant (Zhang et al., 2018). Similar to our results, *gfdB* did not contribute to the osmotic stress

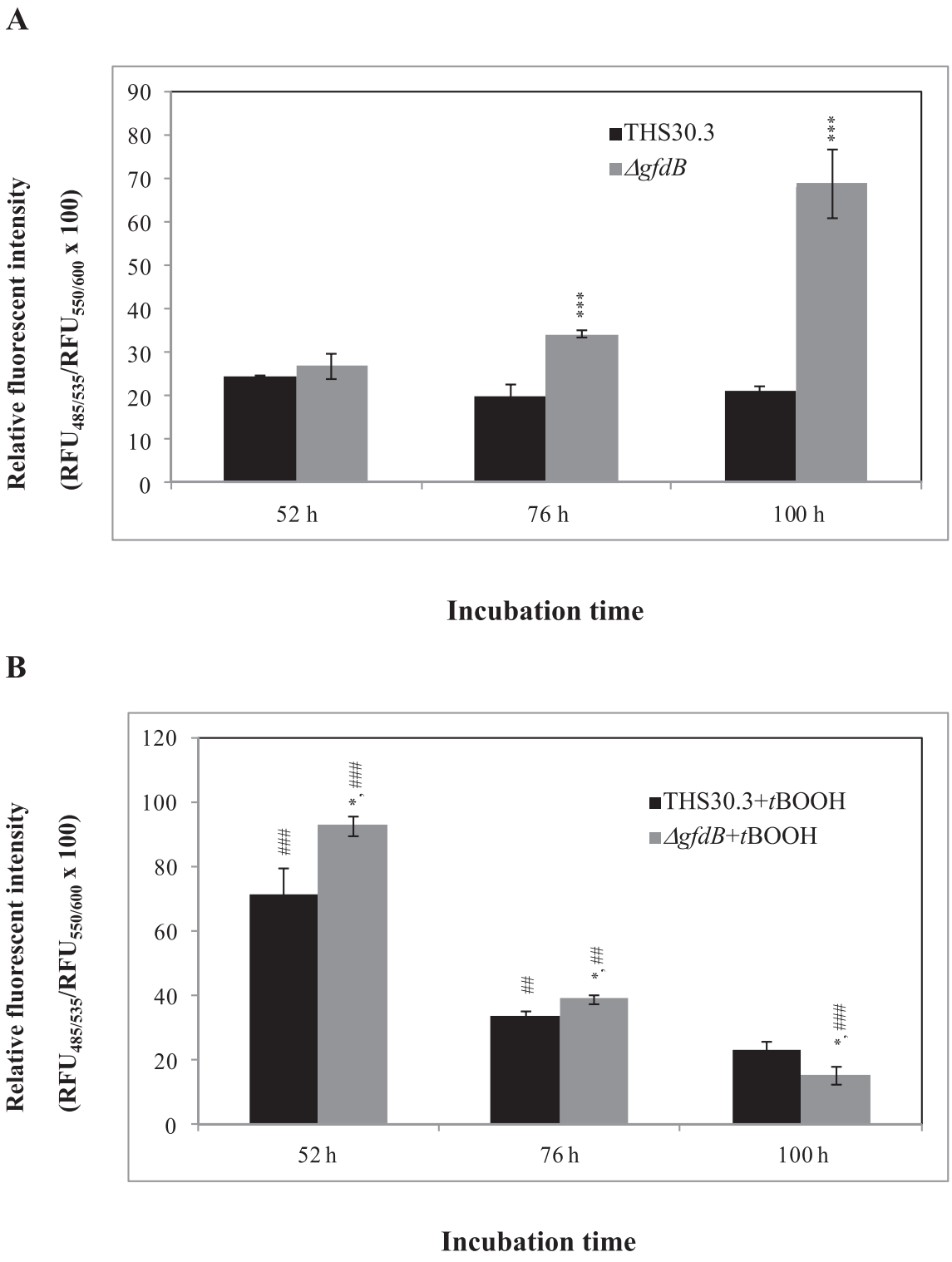

Fig. 3. Comparison of JC-1 fluorescence signal intensity of THS30.3 (control) and *ΔgfdB* without and with *t*BOOH treatment (0.4 mM, added at 20 h incubation). Relative fluorescence intensity (RFU) was calculated by comparing the ratios of green and red fluorescence maxima: $RFU_{485/535}/RFU_{550/600} \times 100$. Incubation times represent the whole time periods since inoculation of the cultures. Relative fluorescence intensity values are shown as mean ± SD calculated from three independent experiments. Significant differences between control and mutant cultures (*, p<5 %, **, p<1 % and ***, p<0.1 %) as well as between untreated and treated (0.4 mM *t*BOOH) cultures (#, p<5 %, ##, p<1 % and ###, p<0.1 %) are also presented.

response in *A. fumigatus* (Zhang et al., 2018) (Table 2) and, hence, the physiological roles including the stress response related functions seem to be partitioned delicately between the paralogous *gfd* genes in the Aspergilli.

Gene duplication events occur frequently in fungal genomes and the new gene pairs will undergo necessarily neofunctionalisation and subfunctionalisation events to avoid harmful imbalanced gene dosages (Papp et al., 2003; Emri et al., 2018). Similar partitioning of

Table 2
Comparison of the phenotypes of *ΔgfdA* and *ΔgfdB* mutants.

	ΔgfdA (Fillinger et al., 2001)	*ΔgfdB*
Parameters tested		
Oxidative stress sensitivity	n.d.	increased
Cell wall stress sensitivity	increased (to Calcofluor White)	increased (to Congo Red)
Osmoremediable growth	increased	decreased
Viability	n.d.	decreased
Apoptosis	n.d.	increased
Alditol content	glycerol	no glycerol mannitol and erythritol (no stress) mannitol (*t*BOOH stress)
Sterigmatocystin production	n.d.	increased

n.d.-not determined.

stress response-related and unrelated physiological functions has been reported for a number of fungal gene duplicates, *e.g.* the *Candida albicans* protein kinase A genes *tpk1* and *tpk2* (Giacometti et al., 2009, 2011), the *S. pombe* TOR kinases *tor1* and *tor2* (Weisman, 2004) and for the *A. nidulans* mitogen activated protein kinase genes *hogA/sakA* and *mpkC* (Pereira Silva et al., 2017; Garrido-Bazán et al., 2018), which are all important signalling pathway elements. It is interesting to see that similarly clear-cut and significant neofunctionalisation and/or subfunctionalisation of genes occurred in the case of the *A. nidulans* primary metabolic genes *gfdA* and *gfdB*. Among the Aspergilli, gene amplification of other primary metabolic genes also took place with allocated tasks. For example, the gene coding for glyceraldehyde-3-phosphate dehydrogenase (*gpd*) was triplicated in *A. clavatus* and *gpdA* was involved in glycolysis meanwhile the paralogs *gpdB* and *gpdC* were expressed only under gluconeogenesis (Flipphi et al., 2009).

There is an increasing body of evidence on that oxidative stress response is connected intimately to secondary metabolite, including mycotoxin, production in filamentous fungi (Leiter et al., 2016). Not surprisingly, because the inactivation of *gfdB* affected clearly the oxidative stress sensitivity of the cells and increased intracellular RS levels the gene deletion also resulted in higher sterigmatocystin productions in both unstressed and stressed (*t*BOOH) cultures (Tables 1 and 2). Our observations are in line with earlier data published in this field, which indicated that (i) alterations of intracellular RS levels influenced the biosyntheses of mycotoxins and (ii) the induction of lipid peroxidation, *e.g.* by *t*BOOH, induced mycotoxin productions (Yin et al., 2013; Reverberi et al., 2008; Roze et al., 2013; Jayashree and Subramanyam, 2000; Wee et al., 2017).

The *ΔgfdB* strain was moderately sensitive to the cell wall integrity stress eliciting agent CongoRed (Fig. 1, Table 2). On the other hand, the elimination of *gfdA* resulted in a marked Calcofluor White (another widely used cell wall integrity disturbing dye) sensitivity and profound changes in hyphal morphology manifested in swollen hyphal tips and shorter hyphal extension (Fillinger et al., 2001). The cell wall stress sensitivity of the *ΔgfdA* strain brought about osmoremediable growth defect in the presence of 1 M NaCl except on glycerol carbon source. No similar phenotypes were observed in the *ΔgfdB* strain (Fig. S3; Table 2) (Fillinger et al., 2001). It is important to mention that such osmoremediation of the growth defect of *ΔgfdA* was not the result of any elevation in the intracellular glycerol-3-phosphate activity. Most likely, this phenotype was a consequence of the activation of signalling pathways stabilizing the integrity of the cell wall (Fillinger et al., 2001).

The viabilities observed in *ΔgfdB* cultures were much lower than those recorded with the THS30.3 control strain which coincided with the earlier onset of programmed cell death (Figs. 2 and 3; Table 2). The intracellular accumulation of RS is one of the major signals leading to apoptosis in eukaryotes including fungal cells (Gonçalves et al., 2017; Leiter et al., 2005). Elevated RS brings about the disintegration of mitochondria, which was demonstrated in our study as well using the JC-1 mitochondrial membrane potential sensitive dye (Fig. 3; Table 2). The significantly elevated apoptotic cell death rates clearly visible in *ΔgfdB* mycelia support the view that GfdB was anti-apoptotic in intact *A. nidulans* mycelium (Figs. 2 and 3; Table 2).

Concerning the production of compatible solutes, Fillinger et al. (2001) measured 20-fold higher glycerol contents in both *ΔgfdA* and wild-type 24 h old mycelia after 1 M NaCl treatment. Importantly, glycerol was also detected in *ΔgfdA* mycelia under unstressed conditions but at lower concentrations than in the control strain (Table 2) (Fillinger et al., 2001).

In our experimental system, no glycerol biosynthesis was detected surprisingly in 44 h submerged cultures of either the *ΔgfdB* or the control strain, which observation coincides with the results of the study of Beever and Laracy (1986), where no glycerol was measured either in late exponential phase submerged cultures of *A. nidulans* without any stress treatment. In our system, the strains produced alditols (mannitol and erythritol) instead of glycerol to maintain their intracellular compatible solute concentration (Fig. S4; Table 2). The inactivation of *gfdB* disturbed the mannitol production of the fungus, while the erythritol content of mycelia remained unchanged in comparison to the control (Table 2). Further studies are needed to answer the exciting questions (i) why alditols are the preferred compatible solutes in ageing cultures, (ii) what sort of carbon sources and intracellular reserves are used to reach and maintain satisfactorily high mannitol and erythritol concentrations and (iii) whether or not GfdB (and also GfdA) play a function in providing the alditol biosynthetic pathways with carbon coming from the oxidation of glycerol. Importantly, the biosynthetic pathways of compatible solutes seem to be organically coupled and interconnected at the level of major primary metabolic pathways like glycolysis, gluconeogenesis and the pentose phosphate pathway (Meena et al., 2015; Rzechonek et al., 2018).

Interestingly, no erythritol production was recorded under *t*BOOH-elicited stress, which may be the consequence of the increased demand for NADPH produced by the pentose phosphate pathway (Varecza et al., 2006), which also the source of erythrose-4-phosphate, the precursor of erythritol (Rzechonek et al., 2018). This hypothesis is supported by the increased specific GR activity found in the *ΔgfdB* cultures (Table 1) because this enzyme is one of the major NADPH consumers in oxidative stress exposed eukaryotic cells (Emri et al., 1997, 1999; Pócsi et al., 2004). Moreover, the deletion of *gfdB* induced nearly twice higher mannitol accumulation in comparison to the control strain meanwhile the mannitol concentration did not change significantly in the THS30.3 control strain (Table 2). This means that *ΔgfdB* hyphae with redox imbalances needed more compatible solutes to stabilize their cell integrity than the hyphae of the control THS30.3 strain. Our observations suggest that the role of *gfdB* seems to be far more than glycerol production in *A. nidulans*.

Several studies have demonstrated that mannitol can substitute glycerol under osmotic stress conditions. Furthermore, mannitol overproduction has also been observed under oxidative stress conditions for example in *Aspergillus niger* (Ruijter et al., 2003), in the human pathogenic fungus *Cryptococcus neoformans* during invasion of the host (Chaturvedi et al., 1996) as well as in the plant pathogenic fungus *Alternaria alternata* during infecting plants

(Jennings et al., 1998). Hence, alditols especially mannitol seem to be a key element of oxidative stress defence as well in fungi.

In the *gpd1Δgpd2Δ* double mutant of *S. cerevisiae*, which lacks both of the glycerol 3-phosphate dehydrogenases, mannitol or sorbitol were overproduced by the heterologous expression of mannitol-1-phosphate dehydrogenase and sorbitol-6-phosphate dehydrogenase biosynthetic genes during osmotic stress but these compatible solutes could only replace glycerol partially (Shen et al., 1999). This means that glycerol seems to be still an indispensable metabolite under exposures to osmotic stress.

5. Conclusions

In summary, our study confirmed that *gfdB*, putatively encoding a glycerol 3-phosphate dehydrogenase, has different physiological functions than those of its paralog *gfdA* in *A. nidulans*. GfdB contributes to the oxidative stress defence, the maintenance of the cell viability and the hindrance of the onset of programmed cell death in this filamentous fungus. Surprisingly, *A. nidulans* overproduced mannitol to mitigate the deleterious effects of oxidative stress initiated by the lipid peroxidising agent *t*BOOH. Our results supported the view that gene pairs coming from gene duplication event will necessarily undergo neofunctionalisation and/or subfunctionalisation processes and, hence, will fulfil different physiological functions with minimal overlaps. Further research should aim shedding light on the physiological functions of other glycerol 3-phosphate dehydrogenase paralog pairs in other Aspergilli.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.09.011.

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