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

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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 (tBOOH), 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 tBOOH, only mannitol was detected in both mutant and control mycelia and the accumulation of mannitol even intensified in the ΔgfdB strain.

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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 Wernher-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, GDP1 and GDP2, have been identified, which encode glycerol 3-phosphate dehydrogenases with different physiological functions. Meanwhile GDP1 is involved in osmoadaptation GDP2 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, GDP1 and GDP2, were identified (Ohmiya et al., 1995). As expected, the
gpd1Δ mutant showed hypersmotic 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 gdp2::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., 2001). 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 hypersmotic 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 found its functions mostly in oxidative (tert-butyl-hydroperoxide (tBOOH), diamide, H2O2) and cell wall integrity (CongoRed) stress defences. Disturbances recorded in the reactive species (RS) productions and in specific antioxidant enzyme (catalase, glutathione peroxidase, superoxide 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 (tBOOH) 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; veAΔ), rRAW16 (pyrG89; yaA2; veAΔ), THS30.3 (pyrG89, AfupyrGΔ; pyroA4; veAΔ) and ΔgfdB (pyrG89; ΔgfdB::AfupyrGΔ; pyroA4++; veAΔ). 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_FSGC_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 (tBOOH, triggering lipid peroxidation), 0.08 mM menadione sodium bisulfite (MSB, increasing intracellular superoxide level) (Pociš 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 x 10⁶ conidia ml⁻¹) and were inoculated 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, AfupyrGΔ; pyroA4; veAΔ) 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 10⁸ spores and incubated at 37 °C and at 220 rpm shaking frequency. Oxidative stress was induced by the addition of 0.4 mM tBOOH 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′-dichlorofluorescein diacetate (Halliwell and Gutteridge, 2007). RS includes all Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), which oxidize 2′,7′-dichlorofluorescein to DCF (Halliwell and Gutteridge, 2007). At the incubation times tested, 10 μM 2′,7′-dichlorofluorescein 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 spectrophuorometrically (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: \(RFU_{535\text{ nm}}/RFU_{600\text{ nm}} \times 100\) (Adám 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 mm, 4.6*150 mm), eluent: 5 mM H₂SO₄ 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 K₂CO₃, 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, tBOOH 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 CdCl₂ (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 tBOOH) 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 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 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 tBOOH [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 tBOOH 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 tBOOH 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
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 rBOOH, sterigmatocystin production decreased in both strains but remained fully the HPLC analysis data concerning the alditol contents of mycelia (Fig. S5).

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

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 rBOOH (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) (Adám et al., 2008). Deletion of the gfdB gene resulted in about a three-fold increase in apoptosis rate after 100 h incubation without any rBOOH 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 synthesized in two consecutive steps in this...
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, \textit{gfdA} and \textit{gfdB}, accommodated by the genome of \textit{A. nidulans} (de Vries et al., 2017). The physiological functions of \textit{gfdA} have been investigated and discussed by Fillinger et al. (2001) and, in this study, we demonstrated that the physiological roles attributable to \textit{gfdB} are remarkably different from those of \textit{gfdA} in \textit{A. nidulans}.

Inactivation of \textit{gfdB} resulted in higher oxidative stress sensitivity compared to the control strain but, in contrast to \textit{gfdA}, no hyperosmotic phenotype was observed, which indicated that \textit{gfdB} was not responsible for the osmotic stability of this fungus. On the other hand, the oxidative stress sensitivity phenotypes of the \textit{ΔgfdB} strain and the higher RS levels manifested in the \textit{ΔgfdB} mutant even under unstressed conditions suggested that \textit{gfdB} should be an important element of the oxidative stress defence of the fungus. Interestingly, the oxidative stress defence system of the \textit{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 \textit{gfdB}. It is important to note that in \textit{A. fumigatus} the inactivation of \textit{gfdB} did not result in decreased growth in glucose-containing medium and the \textit{gfdA} gene could not be substituted functionally by \textit{gfdB} even by over-expressing \textit{gfdB} in the \textit{ΔgfdA} mutant (Zhang et al., 2018). Similar to our results, \textit{gfdB} did not contribute to the osmotic stress.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{MTT.png}
\caption{Changes in the MTT reducing activities of THS30.3 (control) and \textit{ΔgfdB} cultures. The specific MTT reducing activities of the cells was measured spectrophotometrically at 550 nm (\textit{A}_{550}), which are presented as mean ± SD values calculated from three independent experiments. \textit{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 \textit{t}BOOH) cultures (#, \(p<5\%), ##, \(p<1\%\) and ###, \(p<0.1\%\)) are also indicated.}
\end{figure}
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
stress response-related and unrelated physiological functions have 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 while the paralogs gpdB and gpdC were expressed only under gluconeogenesis (Fipphi et al., 2009).

There is an increasing body of evidence on that oxidative stress response is connected intimately to secondary metabolite, including mycoxin, 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 (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 mycoxins and (ii) the induction of lipid peroxidation, e.g. by boOOH, induced mycoxin productions (Yin et al., 2013; Reverberi et al., 2008; Roze et al., 2013; Jayashree and Subramanyam, 2000; Wee et al., 2017).

The gfdB strain was moderately sensitive to the cell wall integrity stress eliciting agent CongoRed (Fig. 1, Table 2). On the other hand, the elimination of gfdB 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 gfdA 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 gfdB strain (Fig. S3; Table 2) (Fillinger et al., 2001). It is important to mention that such osmoremediable, the growth defect of gfdA 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 gfdB 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 (González 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 gfdB 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 gfdA and wild-type 24 h old mycelia after 1 M NaCl treatment. Importantly, glycerol was also detected in gfdA mycelia under un-stressed 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 subcultured cultures of either the gfdB 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 un-stressed or in osmoremediable 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; Rzechonke et al., 2018).

Interestingly, no erythritol production was recorded under 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 (Rzechonke et al., 2018). This hypothesis is supported by the increased specific GR activity found in the gfdB 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 gfdB 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.

### Table 2

| Parameters tested | gfdA | gfdB |
|------------------|------|------|
| **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 | increased mannitol and erythritol (no stress) |
| **Sterigmatocystin production** | n.d. | increased |

n.d.—not determined.
In our study, we 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 BOOH. Our results supported the view that gene pairs coming from gene duplication events will necessarily undergo neofunctionalisation and/or sub-functionalisation processes and, hence, will fulfill 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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### 5. Conclusions

In our study, we 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 BOOH. Our results supported the view that gene pairs coming from gene duplication event will necessarily undergo neofunctionalisation and/or sub-functionalisation processes and, hence, will fulfill 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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