Transcriptional analysis of the response of *Neurospora crassa* to phytosphingosine reveals links to mitochondrial function

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Treatment of *Neurospora crassa* cells with phytosphingosine (PHS) induces programmed cell death (PCD) by an unknown mechanism. To determine the relationship between PHS treatment and PCD, we determined changes in global gene expression levels in *N. crassa* during a time-course of PHS treatment. Most genes having differential expression levels compared to untreated samples showed an increase in relative expression level upon PHS exposure. However, genes encoding mitochondrial proteins were highly enriched among ~100 genes that showed a relative decrease in expression levels after PHS treatment, suggesting that repression of these genes might be related to the death-inducing effects of PHS. Since mutants in respiratory chain complex I are more resistant to both PHS and hydrogen peroxide (H₂O₂) than the wild-type strain, possibly related to the production of reactive oxygen species, we also compared gene expression profiles of a complex I mutant (nuo14) and wild-type in response to H₂O₂. Genes with higher expression levels in the mutant, in the presence of H₂O₂, are also significantly enriched in genes encoding mitochondrial proteins. These data suggest that complex I mutants cope better with drug-induced decrease in expression of genes encoding mitochondrial proteins and may explain their increased resistance to both PHS and H₂O₂. As a way of identifying new components required for PHS-induced death, we analysed the PHS sensitivity of 24 strains carrying deletions in genes that showed a significant alteration in expression pattern when the wild-type was exposed to the sphingolipid. Two additional mutants showing increased resistance to PHS were identified and both encode predicted mitochondrial proteins, further supporting the role of the mitochondria in PHS-induced PCD.

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INTRODUCTION

In response to endogenous or external stimuli, living cells can undergo a genetic programme eventually leading to death, commonly known as programmed cell death (PCD). This suicide process can take several forms (apoptosis,

necrosis, autophagy), each of which displays typical characteristics at both the cellular and molecular level. These processes can be interchangeable and are sometimes difficult to distinguish. In metazoan organisms, the death programme is essential for development and its dysfunction may result in human disease, such as cancer. In addition to multicellular eukaryotic species, both prokaryotic and eukaryotic unicellular organisms also undergo PCD (Cheng *et al.*, 2008; Green & Kroemer, 2004; Hamann *et al.*, 2008; Madeo *et al.*, 2004).

Mitochondria are cellular organelles, still possessing their own genome, that are responsible for the production of most cellular energy in eukaryotes. This production occurs

Abbreviations: PCD, programmed cell death; PHS, phytosphingosine; ROS, reactive oxygen species.

The microarray data associated with this paper have been deposited at the Yale Microarray Experimental Design site (<http://www.yale.edu/townsend/Links/ffdatabase/introduction.html>) under Experiment ID 48.

A supplementary table showing mRNA profiling results and functional annotations is available with the online version of this paper.

mainly in the mitochondrial inner membrane through the process of oxidative phosphorylation, which involves the components of the respiratory chain and ATP synthase (Hatefi, 1985). The involvement of mitochondria and specific mitochondrial proteins, particularly components of the respiratory chain, in most cases of PCD has been well established. This includes, for example, the generation of reactive oxygen species (ROS) and the release of mitochondrial proteins (Eisenberg *et al.*, 2007; Pereira *et al.*, 2008; Skulachev, 2006).

Many of the proteins and mechanisms related to PCD have been intensively studied and characterized. Much less explored has been the analysis of gene expression during the death process (Fedorova *et al.*, 2005; Laun *et al.*, 2005). However, it is becoming increasingly clear that PCD may involve complex metabolic networks and is a more complicated cellular process than initially thought (Kimchi, 2007). These observations imply that many connected gene products remain to be identified. In addition, it is likely that different organisms, especially eukaryotic microbes, may vary in both the mechanism and the suite of responses associated with PCD. Although work in *Saccharomyces cerevisiae* has identified a number of fungal homologues of mammalian 'PCD' genes (Eisenberg *et al.*, 2007; Madeo *et al.*, 2004), filamentous fungi contain additional PCD genes (Fedorova *et al.*, 2005). Filamentous fungi have more similarities to higher eukaryotes (e.g. multicellularity) and thus represent an excellent alternative system to investigate the process. The discovery of novel proteins involved in PCD and their targeting, with, for example, inhibitors, will be very useful for the modulation of cell killing.

We are interested in the overall analysis of gene expression during drug-induced PCD as a means of finding novel proteins and metabolic pathways involved in the process. We use the filamentous fungus *Neurospora crassa* as a model organism because it offers many experimental advantages (Davis & Perkins, 2002; Dunlap *et al.*, 2007) and PCD can be induced by different means (Castro *et al.*, 2008; Glass & Dementhon, 2006; Lu, 2006). Moreover, the ongoing project for the disruption of all predicted genes in the *N. crassa* genome (Colot *et al.*, 2006) allows direct testing of the sensitivity of specific mutants to PCD-inducing stimuli, including mutants in genes identified by expression profiling. In filamentous fungi, the sphingolipid phytosphingosine (PHS) induces PCD with the involvement of mitochondria (Castro *et al.*, 2008; Cheng *et al.*, 2003; Woodcock, 2006). In this article, we present our analysis of the transcriptional response to the death-inducer PHS in *N. crassa* and its major effect on genes encoding mitochondrial proteins. We further show that this type of analysis is useful to identify novel components associated with PCD in filamentous fungi.

METHODS

Growth conditions and experimental design. The wild-type *N. crassa* sequenced strain FGSC2489 (Galagan *et al.*, 2003) and the gene

deletion strains generated by the *Neurospora* Genome Project (Colot *et al.*, 2006) were obtained from the Fungal Genetics Stock Center (FGSC) (McCluskey, 2003). The complex I mutant *nuo14* (Marques *et al.*, 2005), the AMID (Δ NCU06061; FGSC12090) deletion strain (Castro *et al.*, 2008) and the general handling of the fungal strains (Davis & de Serres, 1970) have been described before. For the determination of drug sensitivity in spot assays, conidia from different strains were harvested with distilled water and adjusted to a concentration of 6.56×10^7 cells ml^{-1} . Threefold serial dilutions of each conidial suspension were then spotted on the appropriate GFS plates (Davis & de Serres, 1970) and incubated at 26 °C, as detailed before (Castro *et al.*, 2008).

Conidia for microarrays were obtained from cultures grown for 7 days in Vogel's minimal medium (Davis & de Serres, 1970) at 25 °C under constant light (Kasuga *et al.*, 2005). To study the effects of PHS on gene expression, conidia (10^7 ml^{-1}) were germinated for 5 h in Vogel's minimal medium at 30 °C with strong agitation. Subsequently, PHS was added ($10 \mu\text{g ml}^{-1}$) from a stock solution in ethanol (4 mg ml^{-1}) and the incubation continued for different times. The mycelium was collected by quick filtration, frozen in liquid nitrogen and kept at -70 °C.

For assessing the effect of hydrogen peroxide (H_2O_2) treatment on gene expression profiles, strains were inoculated onto large square Petri dishes that were halved with a barrier. One side was filled with Bird's medium (Metzenberg, 2004), while the other side of the Petri dish contained either Bird's medium or Bird's medium with 4 mM H_2O_2 . The large Petri dishes were then covered with a cellophane sheet. Strains were inoculated onto the first half of the Petri dish and allowed to grow 1 cm into the half of the Petri dish containing no treatment (Bird's medium) or treatment (Bird's medium plus 4 mM H_2O_2). A 1 cm wide cellophane strip containing hyphae at the periphery of the colony was sliced from each plate (treatment and no treatment), frozen in liquid nitrogen and kept at -70 °C.

Closed-circuit designs were employed for microarray comparisons (Fig. 1), because they are statistically robust and provide a higher

Fig. 1. Experimental design for a time-course assessing gene expression patterns of germinating *N. crassa* conidia exposed to PHS. Conidia were germinated in minimal medium for 5 h (time 0) and then incubated in the absence (C) or in the presence of $10 \mu\text{g ml}^{-1}$ PHS in 0.25 % ethanol (P) or ethanol alone (E). Samples were withdrawn at the indicated time points (minutes) and used for microarray hybridization and quantification of gene expression levels. The arrows indicate the di-swap hybridizations, with the arrowhead pointing to the sample labelled with Cy5 (details are described in Methods).

resolution in identifying differentially expressed genes than designs that use a universal reference (Townsend, 2003).

RNA and cDNA processing. RNA was isolated from the fungal mycelia with TRIzol (Invitrogen Life Technologies), purified with the RNeasy kit (Qiagen) and used for the synthesis of cDNA with the Pronto kit (Corning), according to the manufacturers' instructions. The cDNA was labelled with either Cy3 or Cy5 dye (Amersham), dried under vacuum, resuspended in hybridization solution, heated at 95 °C for 5 min and used for hybridization. Each hybridization was duplicated, labelling one cDNA sample with Cy3 and the other with Cy5, and vice versa (di-swap).

Microarray slide hybridization. We used γ -aminopropylsilane slides printed with 70-mer oligonucleotides, which include the 10 526 ORFs predicted in the *Neurospora* genome (Kasuga *et al.*, 2005; Kasuga & Glass, 2008; Tian *et al.*, 2007). The Pronto kit (Corning) was used for hybridization, following the instruction procedures included. Briefly, after presoak and prehybridization steps of the slides to reduce background, the labelled cDNA was laid on the slides covered with a LifterSlip cover glass (Erie Scientific). Hybridization was performed for 16 h at 42 °C, followed by washing steps to remove unbound DNA.

Microarray data acquisition and analysis. The hybridization images were obtained with a GenePix 4000B scanner and the signals were quantified with the GenePix Pro6 software, which automatically flagged low-quality spots. Then, slides were also inspected manually. Spots with a mean fluorescence intensity for at least one of the Cy3 or Cy5 dyes that was greater than the mean background intensity plus three standard deviations were selected for further analysis if less than 0.02% of the pixels were saturated. Normalized ratio data were analysed with the Bayesian Analysis of Gene Expression Levels (BAGEL) software in order to calculate a relative expression level and a credible interval for each gene in each sample (Townsend & Hartl, 2002). The profiles of gene expression were clustered with Hierarchical Clustering Explorer 3.0 (Frishman *et al.*, 2001; Seo & Shneiderman, 2002), using Pearson's correlation coefficient to measure similarities in expression patterns between genes. Consequently, the closest two genes or clusters became successively joined. The genes were associated with functional categories using the FunCat catalogue created by MIPS (Frishman *et al.*, 2001; Ruepp *et al.*, 2004). The statistically significant enrichment of gene groups in these categories relative to all genes with detected mRNA profiles was determined by Fisher's exact test in the statistical software R 2.6 (<http://bioconductor.org>), as detailed by Kasuga & Glass (2008). A significance level of 0.05 was used with multiple testing corrections according to Benjamini & Hochberg (1995). Microarray data have been deposited at the Yale Microarray Experimental Design site (<http://www.yale.edu/townsend/Links/ffdatabase/introduction.html>) under Experiment ID 48. Supplementary Table S1 (available with the online version of this paper) lists mRNA profiling results and functional annotations.

RESULTS

Gene expression in the presence of PHS

PHS induces rapid PCD in *N. crassa* (Castro *et al.*, 2008). In order to analyse the transcriptional response to this drug, we set up the time-course experiment depicted in Fig. 1. Conidia were inoculated into liquid medium and, after a germination period of 5 h (Kasuga *et al.*, 2005), were further incubated in the presence or absence of PHS

for 2 h. Samples were collected at different time points during the 2 h time-course and used to prepare fungal RNA for evaluation of relative expression levels using full-genome *N. crassa* microarrays (Kasuga *et al.*, 2005; Kasuga & Glass, 2008; Tian *et al.*, 2007). In these experiments, we obtained expression data for 6011 genes, representing more than half of the fungal genome (Supplementary Table S1, page 1). In a parallel experiment, we assessed the effects of PHS on cell death by methylene blue staining (Fleissner & Glass, 2007). Nuclei from live cells are not stained by the dye. Upon exposure to PHS, cell death is induced in a time-dependent manner (Fig. 2).

The time-course profiles of gene expression were individually inspected and those genes displaying differential expression in the presence or absence of PHS were selected for further analysis. At least 762 genes (~13%) were influenced by PHS treatment, indicating that an active transcriptional response to drug exposure occurs (Supplementary Table S1, page 2). This set of affected genes was grouped by clustering analysis and the results are shown in Fig. 3. Three typical expression profiles were observed: genes that showed an early increase in relative expression level following exposure to PHS (15–30 min treatment, 272 genes), genes whose relative expression levels increased later following exposure to PHS (60–120 min, 387 genes) and genes that showed a relative decrease in expression level following exposure to PHS (103 genes).

The 762 genes that showed differential relative expression levels when *N. crassa* cells were exposed to PHS were assigned to a functional category, as defined by the FunCat catalogue (Ruepp *et al.*, 2004). We evaluated whether specific functional categories were statistically over-represented in each of the 'early-induced', 'late-induced' and 'repressed' gene categories (Table 1). As could be anticipated as a death-inducer effect, an enrichment for

Fig. 2. Analysis of cell death induced by PHS in *N. crassa*. Conidia were germinated in minimal medium for 3.5 h (time 0) and then incubated in the presence of 10 $\mu\text{g PHS ml}^{-1}$ in 0.25% ethanol (■) or ethanol alone (●). Samples were withdrawn at the indicated time points and the percentage of cell nuclei that stained with 0.003% (w/v) methylene blue (a vital dye) was determined. Standard deviations from three experiments are shown.

Fig. 3. Profile of the time-course of differential gene expression patterns in *N. crassa* germinating conidia upon PHS treatment. The quantification of expression levels was obtained from experiments as described in Fig. 1. Genes that showed differential expression across the time-course in the presence or absence of PHS were clustered according to their similarity of expression patterns. The green and black colours indicate high and low expression levels, respectively.

genes induced early following treatment with PHS are predicted to be involved in cell rescue and stress response, protein degradation and modification, and the glyoxylate cycle, involved in lipid metabolism. Functional categories that included predicted cell cycle and DNA processing proteins, signal transduction and protein fate were enriched among genes that showed a later increase in relative expression level when *N. crassa* was exposed to PHS. However, the most dramatic effect of treatment of *N. crassa* with PHS appears to be a decrease in the expression level of genes encoding mitochondrial proteins, in particular those involved in oxidative phosphorylation. Both nuclear and cytoplasmic genes coding for mitochondrial proteins were highly enriched in the gene set repressed by PHS treatment (Supplementary Table S1,

page 3). The statistical significance of this enrichment can be inferred from the extremely low *P*-values (Table 1).

Comparison of the wild-type and *nuo14* response to H₂O₂

We previously determined that strains containing mutations in complex I were more resistant to treatment with PHS or H₂O₂, although strains containing mutations in other components of the respiratory chain did not show a similar resistance to either treatment (Castro *et al.*, 2008). These observations suggest that the resistance of the complex I mutants to PHS and to H₂O₂ include similar mechanisms. We therefore investigated whether this resistance was associated with differential gene expression.

Table 1. Functional categories over-represented among genes displaying differential expression upon PHS treatment

Description	No. of genes in the cluster		Total no. of genes	P-value*
	Obs†	Exp†		
Early induced (272)				
02.04 Glyoxylate cycle	3	0.3	6	3.97E−02
14 PROTEIN FATE (folding, modification, destination)	36	21	454	2.90E−02
14.13 Protein/peptide degradation	16	5	109	1.33E−03
14.13.01 Cytoplasmic and nuclear protein degradation	7	1.7	36	2.93E−02
14.13.01.01 Proteasomal degradation (ubiquitin/proteasomal pathway)	5	0.9	19	3.35E−02
32 CELL RESCUE, DEFENCE AND VIRULENCE	20	8.8	191	1.82E−02
32.01 Stress response	14	4	86	1.33E−03
Late induced (387)				
10 CELL CYCLE AND DNA PROCESSING	30	16.6	252	3.59E−02
10.03 Cell cycle	21	10.3	156	3.52E−02
14 PROTEIN FATE (folding, modification, destination)	54	29.9	454	5.12E−04
30.05 Transmembrane signal transduction	4	0.6	9	3.84E−02
70.10 Nucleus	29	15.4	234	2.96E−02
Repressed (103)				
02 ENERGY	48	2.8	162	1.44E−46
02.07 Pentose-phosphate pathway	3	0.1	8	9.26E−03
02.11 Electron transport and membrane-associated energy conservation	29	0.9	49	1.14E−37
02.13 Respiration	35	1.2	67	2.38E−43
02.13.03 Aerobic respiration	16	0.4	25	7.04E−21
02.45 Energy conversion and regeneration	3	0.2	13	3.25E−02
02.45.15 Energy generation (e.g. ATP synthase)	3	0.2	10	1.87E−02
20.01 Transported compounds (substrates)	24	4.1	234	7.07E−11
20.01.15 Electron transport	19	0.7	39	7.74E−22
20.03 Transport facilities	16	2.3	131	4.00E−08
70.16 Mitochondrion	44	4.8	274	4.98E−30

*P-values according to Fisher's exact test with Benjamini & Hochberg (1995) multiple testing correction.

†Obs, observed; Exp, expected. 'Exp' is the number of genes predicted to be in a functional category by chance from the total number of genes whose expression profiles were detected versus the expression profile from the number of genes actually observed ('Obs').

We compared the response of wild-type and a complex I mutant (*nuo14*) to treatment with H₂O₂; the resistance of complex I mutants towards H₂O₂ is more marked than their resistance towards PHS (Castro *et al.*, 2008). Our experimental design is illustrated in Fig. 4(A). In these experiments, we obtained microarray signal data for 5709 genes (Supplementary Table S1, page 4).

Although the H₂O₂ concentration used (4 mM) was lethal to conidia (data not shown), the morphological response of *N. crassa* mycelia to H₂O₂ treatment was relatively mild. The expression of catalase-3 is elevated 1.5–2-fold in response to H₂O₂ (Fig. 4B). This protein is predicted to be exported from cells and is thus likely to be involved in the removal of the H₂O₂ present in the culture medium. In the gene set from either wild-type or *nuo14* that showed altered expression profiles in response to H₂O₂, we did not observe significant changes in the expression level of other detoxifying enzymes, such as other catalases, peroxidases, mitochondrial and cytosolic superoxide dismutases, thioredoxins or enzymes acting on glutathione. Unexpectedly, the mRNA levels of mitochondrial alternative oxidase

(*NCU07953.2*) and cytochrome *c* (*NCU01808.2*) were 2–2.5-fold higher in the complex I mutant grown in minimal medium as compared to wild-type, reminiscent of the situation in complex IV (Bertrand & Pittenger, 1972; Descheneau *et al.*, 2005) and complex III mutants (Duarte & Videira, 2009). Induction of alternative oxidase is expected in complex IV and complex III mutants, but not in complex I mutants, because it represents an alternative pathway of electron flow that branches off the standard electron-transport chain after complex I, but before complexes III and IV. These results suggest that induction of alternative oxidase and cytochrome *c* represent a general response to deficiencies in the mitochondrial respiratory chain.

Perhaps the most interesting result from the data analysis is depicted in Fig. 4(C). We considered the gene set that showed a statistically significant ($P < 0.05$) increased expression level in the complex I mutant as compared to wild-type in response to H₂O₂. The analysis of the distribution of these genes by functional category revealed that genes encoding mitochondrial proteins were the most

Fig. 4. Comparison of gene expression between wild-type and a complex I mutant in the presence or absence of H₂O₂. (A) Scheme of the microarray hybridizations. A mycelial strip from the wild-type strain and the nuo14 mutant that had grown into solid minimal medium (MM) or into minimal medium containing 4 mM H₂O₂ was used to compare gene expression levels. (B) Relative expression levels of catalase-3 (*NCU00355.2*). (C) Venn diagram showing the number of genes with statistically significant higher expression (↑) in either wild-type or the nuo14 mutant in each of the different growth conditions. A functional category with statistically significant over-representation in a group of genes in the nuo14 mutant exposed to H₂O₂ is indicated.

over-represented category within the group. These results suggest that complex I mutants may cope better than wild-type with the induced repression of genes encoding mitochondrial proteins upon exposure to PHS (as described above for the PHS effects on wild-type), and this may explain why they are more resistant to both PHS and H₂O₂.

Sensitivity of selected deletion mutants to PHS

The expression of many *N. crassa* genes is affected by PHS exposure, as described above. The cellular processes in which these genes participate might therefore be relevant for PHS-induced PCD. Consequently, mutant strains

carrying deletions in relevant genes might respond differently to PHS than the wild-type strain. To verify this, we took advantage of an ongoing project to disrupt all *N. crassa* genes (Colot *et al.*, 2006; Dunlap *et al.*, 2007) and tested the sensitivity to PHS of several available deletion mutants in spot assays. Serial dilutions of conidia from the different strains were exposed to media containing PHS (Fig. 5). In these experiments, the complex I mutant nuo14 and a mutant lacking an AMID-like protein (*ΔNCU06061.2*) were used as controls. The nuo14 mutant is more resistant to PHS, while *ΔNCU06061.2* is more sensitive to PHS than the wild-type strain (Castro *et al.*, 2008). Among the 24 mutants carrying deletions in genes whose expression is affected by PHS, we identified two strains that were more resistant to the drug than the wild-type strain (Fig. 5). The *NCU00502.2* and *NCU03415.2* genes encode subunit 4 of mitochondrial ATP synthase and a probable aldehyde dehydrogenase, respectively. Our results indicate their involvement in PHS-induced PCD and point to directions for future work.

DISCUSSION

We are interested in characterizing PCD mechanisms in *N. crassa* and in identifying unknown pathways involved in the process. In this work, we performed a detailed time-course analysis of gene expression in the wild-type following exposure to the death-inducing drug PHS. Most genes displaying differential expression upon the drug treatment increased in expression level, showing that the response to PHS treatment induces a transcriptionally active process. Among this set of genes, we found enrichment for genes encoding proteins belonging to functional categories that have been described to participate in stress and death responses (Bonawitz *et al.*, 2006; Fedorova *et al.*, 2005; Fröhlich *et al.*, 2007; Hamann *et al.*, 2008; Laun *et al.*, 2005; Lu, 2006; Lucau-Danila *et al.*, 2005). For instance, genes encoding proteins predicted to be involved in cell rescue and stress response, cell cycle and DNA processing, protein fate and modulation and for those involved in protein degradation were significantly enriched. However, the most striking effect of PHS appears to be the repression of genes encoding mitochondrial proteins, particularly those associated with energy conversion. Both nuclear and mitochondrial genes encoding the organelle proteins were extremely enriched among the ~100 genes that showed a decrease in relative expression following PHS exposure. This is in line with the findings that mitochondria have a central role in PHS-induced PCD (Castro *et al.*, 2008; Cheng *et al.*, 2003; Woodcock, 2006).

Altered transcription during PCD has been noticed before. Nuclear genes encoding mitochondrial proteins represented the largest group found to be differentially regulated in apoptotic yeast (Laun *et al.*, 2005). A reduction of yeast lifespan is associated with deficiencies in mitochondrial gene expression, which result in ROS-mediated inhibition of respiration (Bonawitz *et al.*, 2006). A decrease in

Fig. 5. Sensitivity of selected deletion strains to PHS. Serial threefold dilutions of conidia from the indicated strains were spotted on GFS medium containing $7.5 \mu\text{g PHS ml}^{-1}$ and incubated at 25°C . The less concentrated spots contain ~ 50 conidia. The sensitivity to PHS of other strains tested ($\Delta\text{NCU01376.2}$, $\Delta\text{NCU01640.2}$, $\Delta\text{NCU00355.2}$, $\Delta\text{NCU098-82.2}$, $\Delta\text{NCU04421.2}$, $\Delta\text{NCU07121.2}$, $\Delta\text{NCU06953.2}$, $\Delta\text{NCU03241.2}$, $\Delta\text{NCU047-37.2}$, $\Delta\text{NCU06664.2}$, $\Delta\text{NCU01754.2}$, $\Delta\text{NCU06660.2}$, $\Delta\text{NCU01645.2}$, $\Delta\text{NCU004-61.2}$, $\Delta\text{NCU05627.2}$, $\Delta\text{NCU04635.2}$, $\Delta\text{NCU00865.2}$, $\Delta\text{NCU03888.2}$) was similar to that of the wild-type (not shown).

mitochondrial gene transcripts has also been observed in patients suffering complex I deficiency (van der Westhuizen *et al.*, 2003) and alterations in mitochondrial components were identified in a mouse model of Friedreich ataxia (Coppola *et al.*, 2006). Here we describe, to our knowledge for the first time, a generalized downregulation of genes coding for mitochondrial proteins in response to treatment with PHS. This treatment likely creates an imbalance of mitochondrial components, eventually leading to mitochondrial dysfunction and triggering PCD. It is possible that the signalling for this regulation is mediated by ROS produced by the respiratory chain following drug exposure. First, ROS are known to regulate gene expression (Lucau-Danila *et al.*, 2005) and are elevated in most cases of PCD (Robson, 2006; Skulachev, 2006). Second, we found that mutants in respiratory chain complex I are more resistant to PHS-induced death than the wild-type strain. In a previous study, resistance was correlated with a lower amount of ROS production in complex I mutants in the presence of the sphingolipid PHS (Castro *et al.*, 2008). In the present study, we further compared gene expression patterns between a complex I mutant and wild-type *Neurospora*, including their responses to H_2O_2 , which mimics elevated ROS generation (Bonawitz *et al.*, 2006). We found that genes encoding mitochondrial proteins were the most enriched category among genes that showed increased expression levels in the complex I mutant as compared to wild-type in the presence of H_2O_2 . This may be the reason why complex I mutants are more resistant than wild-type to PHS treatment and it supports our proposition that repression of genes encoding mitochondrial proteins is a crucial event of PHS-induced PCD. The complex I mutants are less affected in the expression of mitochondrial proteins and produce lower amounts of ROS; they may therefore be less prone to undergo PCD.

Our hypothesis was that new genes involved in PHS-induced death would be identified by transcriptional profiling. We therefore tested the PHS sensitivity of available mutants (Colot *et al.*, 2006; Dunlap *et al.*, 2007; McCluskey, 2003) that carried deletions in genes whose

expression was affected (either increased or decreased) by exposure to PHS. Germinating conidia from most of them displayed no altered sensitivity to PHS. It is possible that the missing proteins are involved in PCD, but their role is redundant and can be complemented by other cellular proteins. However, we identified two deletion strains that displayed increased resistance to PHS compared with the wild-type strain, indicating that the absent proteins are involved in PCD. One of the deleted genes encodes a probable aldehyde dehydrogenase. The gene deleted in the second strain codes for a subunit of mitochondrial ATP synthase, connected with the respiratory chain. The identification of increased PHS resistance in these mutants is in line with our findings that mutants containing deletion mutations in genes encoding subunits of respiratory chain complex I also display increased resistance to PHS (Castro *et al.*, 2008). Thus, the analysis of differential gene expression should prove very useful for the identification of novel components/pathways involved in PCD and for the design of drugs acting in the modulation of the process.

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