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Transcriptome profiling of the fungus *Aspergillus nidulans* exposed to a commercial glyphosate-based herbicide under conditions of apparent herbicide tolerance

Robin Mesnage[a,b] , Nathalie Oestreicher[c] , Florence Poirier[d], Valérie Nicolas[e], Céline Boursier[e], Christian Vélot[b,c,*]

[a] Gene Expression and Therapy Group, King's College London, Faculty of Life Sciences & Medicine, Department of Medical and Molecular Genetics, 8th Floor, Tower Wing, Guy's Hospital, Great Maze Pond, London SE1 9RT, United Kingdom

[b] CRIIGEN, 42 rue de Lisbonne, 75008 Paris, France

[c] Equipe VEAC, Université Paris-Sud, Faculté des Sciences, Bât. 350, Avenue Jean Perrin, 91405 Orsay, France — Pôle Risques MRSH-CNRS, EA2608, Université de Caen, Esplanade de la Paix, 14032 Caen, France

[d] Université Paris 13, UFR SMBH, Plateforme PPUP13, 1 rue de Chablis, 93017 Bobigny cedex, France

[e] UMS-IPSIT, US31 Inserm-UMS3679 CNRS, Plateformes Trans-Prot et d'Imagerie Cellulaire, Université Paris-Sud, Faculté de Pharmacie, Tour E1, 5 Rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France

* Corresponding author : Laboratoire VEAC, Université Paris-Sud, Faculté des Sciences, Bât. 350 – RdC - Avenue Jean Perrin, 91405 Orsay, France - Phone : (33) 1 69 15 82 95 – Fax : (33) 1 69 15 61 43

E-mail address : christian.velot@u-psud.fr

E-mail addresses of other authors:

robin.mesnage@kcl.ac.uk; nathalie.oestreicher@u-psud.fr; florence.poirier@univ-paris13.fr; valerie.nicolas@u-psud.fr; celine.boursier@u-psud.fr

Abstract

Glyphosate-based herbicides, such as Roundup®, are the most widely used non-selective, broad-spectrum herbicides. The release of these compounds in large amounts into the environment is susceptible to affect soil quality and health, especially because of the non-target effects on a large range of organisms including soil microorganisms. The soil filamentous fungus *Aspergillus nidulans*, a well-characterized experimental model organism that can be used as a bio-indicator for agricultural soil health, has been previously shown to be highly affected by Roundup GT Plus (R450: 450 g/L of glyphosate) at concentrations far below recommended agricultural application rate, including at a dose that does not cause any macroscopic effect. In this study, we determined alterations in the transcriptome of *A. nidulans* when exposed to R450 at a dose corresponding to the no-observed-adverse-effect level (NOAEL) for macroscopic parameters. A total of 1,816 distinct genes had their expression altered. The most affected biological functions were protein synthesis, amino acids and secondary metabolisms, stress response, as well as detoxification pathways through cytochromes P450, glutathione-S-transferases, and ABC transporters. These results partly explain the molecular mechanisms underlying alterations in growth parameters detected at higher concentrations for this ascomycete fungus. In conclusion, our results highlight molecular disturbances in a soil fungus under conditions of apparent tolerance to the herbicide, and thus confirm the need to question the principle of "substantial equivalence" when applied to plants made tolerant to herbicides.

Keywords

Glyphosate-based herbicide; *Aspergillus nidulans*; Transcriptomics; Apparent herbicide tolerance; Substantial equivalence

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1. Introduction

Glyphosate-based-herbicides (GBH)[1] are the most globally used herbicides in the world. Glyphosate (GLY) was patented as an herbicide active ingredient in 1971 (U.S. Patent No 3,799,758), and the first GBH were rapidly introduced onto the pesticides market in 1974 under the well-known trade name "Roundup®". In 2014, the amount of GBH sprayed by farmers was equivalent to 0.53 kg of GLY per ha on all cropland worldwide (Benbrook, 2016).

Intensive use of GBH has been shown to undermine soil quality by increasing leaching of banned remnant pesticides (Sabatier et al., 2014). GBH application resulted in a substantial decrease in the abundance of soil microorganisms (Santos et al., 2006; Weaver et al., 2007), and especially in toxic effects on symbiotic mycorrhizal fungi (Zaller at al., 2014) as well as on fungi from the genus *Aspergillus* (Carranza et al., 2014; Nicolas et al., 2016) or *Mucor* (Mandl et al., 2018).

In plants, GLY disrupts the shikimate pathway by inhibiting the binding of phosphoenolpyruvate on the active site of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). This causes a shortage in essential aromatic amino acids, and consequently a blockage of protein synthesis. Since many microorganisms such as bacteria and fungi also rely on the shikimate pathway, and thus on EPSPS enzyme function, they represent other potential GBH targets. The inhibition of EPSPS by glyphosate in microorganisms is also considered to be a possible mode of action for the therapy of pathogenic infections provoked by *Toxoplasma gondii*, *Plasmodium falciparum* (the parasite that causes malaria) and *Cryptosporidium parvumb* (U.S. Patent No *7771736 B2*). Glyphosate also proved to be an effective fungicide. The application of GBH on Roundup-tolerant soybeans and wheat suppresses rust diseases caused by *Phakopsora pachyrhizi* and *Puccinia spp.,* respectively (Feng et al., 2005). This was attributed to an inhibition of fungal EPSPS

[1] Abbreviations : GBH, Glyphosate-based herbicide; GLY, Glyphosate; R450, Roundup® "GT Plus"

based on the observation that rust control was proportional to GLY tissue concentrations (Feng et al., 2005), and because shikimate levels were increased with GBH treatments (Nandula, 2010).

However, the existence of the shikimate pathway in microorganisms does not necessarily imply that GBH toxicity would be due to EPSPS inhibition. Although all plant EPSPS are GLY-sensitive (class I), those from microorganisms belong either to class I or to class II (GLY-tolerant) (Funke et al., 2009). In the latter case, toxic effects can only be explained by other targets of GLY and/or surfactants present in the commercial formulations. GBH generally include co-formulants, which are added to increase GLY penetration in plant tissues (Mesnage et al., 2019). These additives are considered as inert although GBH formulations have been shown to be much more toxic than GLY alone in different models (Mesnage et al., 2014; Mesnage et al., 2015), including in microorganisms (Braconi et al., 2006; Clair et al., 2012; Lipok et al., 2010; Nicolas et al., 2016; Qiu et al., 2013). Some of the co-formulants alone are sometimes more toxic than GLY itself (Defarge et al., 2016; Mesnage et al., 2013). The multiplicity of targets for the active principle and/or its co-formulants is reflected by the diversity of the reported GBH toxic effects, which can vary from one organism or cell type to another (Mesnage et al., 2015; Nicolas et al., 2016).

Soil microbes are responsible for many soil processes, including transformation of organic matter and nutrient release, and microbial activity is expected to be one of the most efficient biological parameters to assess soil quality and health as affected by agricultural practices (Anderson, 2003; Beneditti and Dilly, 2006). The well-characterized soil ascomycete fungus *Aspergillus nidulans*, used for decades as an experimental model organism in basic and industrial microbiology research (Martinelli and Kinghorn, 1994), provides a relevant marker for agricultural soil health. We found that the GBH commercial formulation "Roundup GT Plus" containing 450 g/L of GLY (R450) provokes multiple toxic effects affecting various biological processes at doses far below the recommended agricultural application rates, including a dose corresponding to the no-observed-adverse-effect level (NOAEL) for macroscopic parameters (Nicolas et al., 2016).

To get further insights into the molecular processes of these toxicological effects, we supplemented our previous proteomic analysis (Poirier et al., 2017) by a global transcriptome profiling of the fungus *Aspergillus nidulans* exposed to R450 at above-mentioned NOAEL (0.007%, i.e. containing 31.54 mg/L GLY among adjuvants), using a custom microarray (Deloménie et al., 2016). Transcriptome profiles carry the molecular signature of toxic effects, which can be used as biomarkers to study the impacts of pesticides on soil microbiology (Oh et al., 2008; Yu et al., 2006).

GLY is frequently found in agricultural topsoils of the European Union at median values ranging from 0.05 to 1.14 mg/kg among 11 EU countries (Silva et al., 2018). Both glyphosate and its major breakdown product, aminomethylphosphonic acid (AMPA), had a maximum concentration of 2 mg/kg. Glyphosate concentrations were comparable in different US regions with median and maximum concentrations of 9.6 and 476 µg/kg, respectively (Battaglin et al., 2014). In agro-productive areas of the pampas region in Argentina, glyphosate was found at concentrations ranging from 102 to 323 µg/kg (Alonso et al., 2018). However, concentrations may vary considerably depending on many factors (Aparicio et al. 2013; Lupi et al. 2015), including climate (especially rains occurring after application), soil quality and structure, and agricultural management practices. Thus, another Argentinean study carried out in the Mesopotamic Pampas agro-ecosystem reported higher glyphosate concentrations (2,3 ± 0.48 mg/kg), with a maximum concentration reaching 8.1 mg/kg (Primost et al., 2017). Moreover, mainly GLY and AMPA are generally investigated in GBH-treated soils. However, it should be borne in mind that GLY is never used alone but in formulation with surfactants which can also be found in environmental samples. In a study performed by the U.S. Geological Survey, a type of POEA (polyoxyethylene (15) tallow amine) was found in all 21 field samples analyzed which indicates that the potential problems associated with the occurrence of surfactant included in pesticide formulations should not be neglected (Tush et al., 2016). So, it is crucial here to test the effects of a commercial GBH formulation and not of GLY alone. Admittedly, our study uses doses substantially higher than those mentioned above. However, *A. nidulans* is not

from the rhizosphere but is a surface fungus. Therefore, it is exposed to the sprayed doses (between 4.5 and 9 g/L of GLY, among adjuvants), which are much higher than those found in the soil because the latter result from dispersion and dilution effects (Perruzo et al. 2008).

Thus, although the objective of the present work was primarily to better appreciate the extent of the R450 exposure impacts in *A. nidulans* under conditions of apparent herbicide tolerance, (*i.e.* at a dose that does not cause any macroscopic effect), our findings could be used in environmental studies aimed to better understand the adverse effects of GBH in soil microorganisms as a whole. For instance, future studies could use meta-transcriptomics approaches to ensure that the gene networks having their expression altered in our study are also altered in the context of agricultural soils.

2. Materials and Methods

2.1. A. nidulans *strain and growth conditions*

Two independent sets of the *A. nidulans* CV125 (*pabaA1*) strain (Nicolas et al., 2016) liquid cultures were carried out in 400 ml minimal medium (Cove, 1966) with fructose (0.1%) as the carbon source and urea (5 mM) as the nitrogen source, in the absence ("Control") or presence ("Roundup") of the herbicide commercial formulation called "GT plus" (available on the market: homologation 2020448, Monsanto, Anvers, Belgium). "GT Plus" (R450) contains 450g/L of GLY (corresponding to 100%), and the dose used in the "Roundup" samples was 0,007% (i.e. containing 31.5 mg/L of GLY among adjuvants), corresponding to the no-observed-adverse-effect level (NOAEL) associated to macroscopic parameters in *A. nidulans* (Nicolas et al., 2016; Poirier et al., 2017). Liquid cultures were incubated at 30°C for 15 h in an orbital shaker at 150 rpm. For each set and each condition (absence or presence of Roundup), cultures were carried out in quadruplicate.

2.2. Extraction and purification of total RNA

Mycelium from each *A. nidulans* liquid culture was harvested by filtration through sterile Blutex nylon tissue, washed with water, wrung dry, and ground with a pestle in a mortar under liquid nitrogen to a fine powder. First, proteins were denatured in presence of guanidine 6 M, sodium acetate 20 mM (pH 5) and dithiotreitol 1 mM. After a 15 min centrifugation at 10000 rpm at 4°C, supernatant was filtered to remove cell debris, and RNA was precipitated by 2-hours incubation in 2,5 volumes of ethanol 95% at -20°C. After a 10 min centrifugation, the pellet was dissolved in guanidine 6 M, sodium acetate 20 mM (pH 7) and dithiotreitol 1 mM, then precipitated as above. Finally, after another 10 min centrifugation at 10 000 rpm at 4°C, the RNA was dissolved in 500 µL of DEPC-treated sterile water. RNA extract was stored at -20°C (until use) in 1.5 ml of ethanol 70% containing sodium acetate 100 mM. Further purification was carried out from 25% (volume) of each alcoholic RNA extract, by centrifugation followed by three washes of the pellet with 75% ethanol before solubilization in 250 µL RNase-free water. Finally, RNA samples were treated with 900 µL TRIzol® (Life Technologies), according to the manufacturer's instructions, to eliminate possible remaining impurities. After precipitation in 500 µL isopropanol at room temperature, RNA was pelleted by centrifugation, the pellet washed twice with 75% ethanol, then solubilized in 100 µL RNase-free water and stored at -80°C until molecular analyses. RNA purity and quantity was assessed by UV measurement using a BioMate 3S UV-Visible (Thermo Scientific™) spectrophotometer. To quantify the genomic DNA fraction in purified RNA, a qPCR assay using GAPDH gene-targeted primers (Forward: CGACAACGAGTGGGGTTACT; Reverse: GGCATCAACCTTGGAGATGT) was carried out. DNA contaminant was lower than $5x10^{-4}$ in total RNA and lower than $2x10^{-5}$ in labelled cRNA prepared for microarray hybridization. RNA integrity evaluated by capillary electrophoresis using RNA 6000 Nano chips and the 2100 Bioanalyzer

(Agilent Technologies) revealed RIN (RNA Integrity Number) value of 6,13 ± 0.26. Such a value was lower than the RIN threshold usually considered as acceptable (Imbeaud et al., 2005). RNA integrity was confirmed by a further evaluation based on two metrics, i.e. 28S/18S ratio (1,63 ± 0.13) and ΔΔCq 5' vs 3' ratio (<1). The latter was measured upon oligo-dT primed reverse-transcribed RNA with ACTIN 3' (Forward: GTACGATGAGAGCGGTCCTT; Reverse: CAGAAAATACGCGACAACGA) and ACTN 5' (Forward: TCCTCCTCCCTTCCTTACCTG; Reverse: ACATACCCGAACCATTGTCGA) primers (Imbeaud et al., 2005; Vermeulen et al., 2011).

2.3. Gene expression profile analysis

2.3.1. Hybridization of microarrays

A global gene expression analysis was performed from 2 biological replicates of 4 Roundup-treated or -untreated cultures, using custom 8 x15k *A. nidulans* whole genome oligo microarrays (AMADID, Agilent Technologies) designed with eArray (Delomenie et al., 2016). Target preparation and hybridization were carried out according to the manufacturer's instructions. Briefly, 200 ng of total RNA was labelled using the Low Input Quick Amp Labeling Kit, 2 colors (Agilent Technologies): Cy5-CTP for the reference RNA (consisting of a mixture of all samples), and Cy3-CTP for individual samples. Internal standards were derived from the Two-Color RNA Spike-in Kit (Agilent Technologies). The labelled targets were purified using the RNeasy® Mini Kit (Qiagen) and their quality and quantity were confirmed by spectrophotometry and the Bioanalyzer 2100 technology (Agilent Technologies). Equal quantities (325 ng) of Cy3- and Cy5-labeled cRNA targets were mixed and incubated on microarray slides at 65°C for 17 h. After washing, slides were scanned using the Microarray Scanner (G2565CA, Agilent Technologies) at 5-µm resolution and at high and low photomultiplier voltages to optimize the dynamic range of image quantification. The data were extracted from these images using the Agilent Feature Extraction 10.7.3.1. software.

2.3.2. Data processing and statistical analysis of microarray data

Data were computed using the Genespring GX version 12.6 (Agilent Technologies), a web-based tool that allows integrated analysis of two-colour microarrays. Arrays were background corrected using minimum method (any intensity which is zero or negative after background subtraction is set equal to half the minimum of the positive corrected intensities for that array), and a within-array loess normalization was performed. Finally, microarray data were normalized using quantile normalization. Log intensity ratios for each spot were obtained with background subtraction. Systematic biases were corrected by applying successively an intensity dependent print–tip loess normalization (Smyth and Speed, 2003) and a scale between-array normalization (Yang et al., 2001; Yang et al., 2002). A filtering procedure excluded data points that were considered unreliable as they corresponded to probe sets associated with low signal intensities or bad quality features. For the differential analysis, empirical Bayes moderated t-statistics were computed on the log intensity ratios for each gene. Duplicate spots printed on the arrays were considered as repeated measures. All p-values obtained were adjusted by the Benjamini and Hochberg false discovery rate step-up method (Benjamini and Hochberg, 1995) to account for multiple testing. Differentially expressed genes were defined as those whose p-value was statistically significant at a level of $p<0.01$, with absolute fold-change at least >1.5, and for which the mean intensity of the two channels was higher than 5.5 on the $\log_2$ scale. The dye effect was included in the analysis.

2.3.3. Functional analysis

Gene lists obtained from microarrays were included in an enrichment analysis in order to find functional groups highly represented within modulated genes. Fisher's exact test was used in DAVID bioinformatics resources 6.7 tool (http://david.abcc.ncifcrf.gov/) from Entrez Gene IDs to detect a significant over-representation or enrichment of a given group, with $\alpha=0.05$ (Dennis et al., 2003).

Hierarchical clustering was carried out to cluster among the gene signal intensities and among the samples with Omics Explorer 3.0 (Qlucore) from selected enriched gene clusters. Results leading to "hypothetical proteins" were submitted to the Broad Institute database (http://www.broadinstitute.org) as well as to the *Aspergillus* genome database (http://www.aspergillusgenome.org; Cerqueira et al., 2014). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE95241.

2.3.4. Comparison between transcript and protein levels

Fold changes for transcripts and their corresponding proteins measured in our previous study (Poirier et al., 2017) were analysed using R version 3.6.1. The correlation between the changes in the proteome and the transcriptome were studied using a Pearson correlation with R in-house functions. These changes were visualised using the packages *ggplot2* and *ggrepel*.

2.4. RT-qPCR analysis

For quantification of mRNA expression, first strand cDNA was synthesized by reverse-transcription from 1 µg of total RNA, with random hexamers and oligo-dT priming using the iSCRIPT enzyme (Bio-Rad), according to the manufacturer's instructions. PCR primer pairs specific to 28 target and 6 reference genes (Supplemental Table S1) were designed using Primer3Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Untergasser et al., 2007) or Primer-BLAST (Ye et al., 2012) tools. The cDNA synthesized from 4 ng of total RNA was amplified in a CFX96 real time thermal cycler (Bio-Rad) using the SSoADV Univer SYBR® Green Supermix (Bio-Rad) reagent according to manufacturer's instructions, with 500 nM (final concentration) of each primer. Reactions (10 µl) were carried out in duplicate, by 45 two-step cycles (95°C 5 s; 61°C 10 s).

Samples that did not stand the reverse transcription step ("No RT" controls) were amplified on all genes to control for genomic DNA contamination, and melting curve analysis was performed to assess the purity of the PCR products. PCR efficiencies calculated for each gene, from the slopes of calibration curves generated from the pool of all cDNA samples, were above 90%. GeNorm in qBase PLUS tool (Vandesompele et al., 2002) was used to select *actA* and *gpdA* genes as references for normalization of mRNA expression results. The normalized relative expression of target genes in samples was determined using the ΔΔCq method with correction for PCR efficiencies (Pfaffl, 2001), where $NRQ = E_{Target}^{\Delta Cq\ Target} / E_{Ref}^{\Delta CqRef}$ and $\Delta Cq = Cq_{sample} - Cq_{calibrator}$ (Hellemans et al., 2007). Final results were expressed as the *n*-fold differences in target gene expression in treated ("Roundup") vs untreated ("Control") mycelia. Mean values ± SD were obtained from three independent experiments.

2.5. Statistical analysis for RNA and RT-qPCR data

Roundup-treated and control groups were compared for relative transcripts levels with GraphPad Prism® by Student's t-test with a Fischer's test for variance comparison and Welch's correction if unequal variances were found between groups. $P<0.05$ was considered significant. Fieller's theorem was used to calculate the 95% confidence interval (error bars) of the fold changes. Statistics of correlation between fold-changes obtained by RT-qPCR and microarrays were analyzed (http://vassarstats.net/).

3. Results and discussion

In a previous study on the toxicity of Roundup "GT-Plus" in *A. nidulans* (Nicolas et al., 2016), we determined the lowest- and no-observed-adverse effect levels (LOAEL and NOAEL). The determination of these specific doses was done in regard to growth characteristics (growth rate, germination lag time and ratio) and morphology (mycelial organization, pigmentation), both in solid and liquid media. Consistently with our previous proteomic study (Poirier et al., 2017), all the transcriptomic analyses described in the present investigation were performed at the NOAEL dose (0.007%, i.e. containing 31.54 mg/L GLY among adjuvants) for macroscopic parameters.

3.1. Wide-scale transcriptomic analysis

In order to evaluate the molecular effects of Roundup at a dose causing no visible effect, RNA extracted from R450-treated and -untreated *A. nidulans* cultures were subjected to a global gene expression profiling using a custom agilent microarray (8 × 15 K format), for which the reproducibility, specificity and sensitivity was described previously (Deloménie et al., 2016). The effects of R450 were first visualized by plotting each sample as a point in the Cartesian space defined by the three principal components from a principal component analysis (PCA) of gene expression data (Fig. 1A) to reduce the 15,124-dimensional state space (variations in the expression levels of the 15,124 *A. nidulans* genes). The first component explained 43% of data dispersion and separated the group of R450-treated samples from the control group. Statistical comparisons made using p-values obtained by t-tests and adjusted by the Benjamini and Hochberg procedure identified an alteration in gene expression ($p<0.01$, fold-changes>1.5) under R450 treatment for 2,395 probes, corresponding to 1,816 distinct transcripts (since one gene can be represented by multiple probes). They included a comparable number of down-regulated (894; 49%) and up-regulated (922; 51%) genes. All the 2,395 p-values were plotted along with their respective fold changes in the form of a volcano plot (Fig. 1B). The 20 most highly up- and down-regulated genes are listed in Table 1.

In order to ensure confidence in these results, a set of 28 genes was selected among those differentially expressed according to microarray hybridization, and were subjected to RT-qPCR analysis. This allowed to confirm differential expression for 20 (i.e. 71%) of these genes (Fig. 2A). Only the *catA* gene displayed opposite results with the two methods. A highly significant correlation (r = 0.96) between fold-changes measured by microarray analysis and RT-qPCR was observed (Fig. 2B). Overall, the RT-qPCR experiment confirmed the results measured on the microarrays.

We next conducted an ontology analysis of the 1816 genes deregulated by the Roundup treatment using the DAVID gene functional classification tool. A total of 1547 genes were recognized. An enrichment analysis was performed in order to reveal the most affected biological functions using annotations from KEGG, InterPro and Gene Ontology (GO) databases. Fold enrichments (FE) and p-values (p) for the most affected pathways are presented in Fig. 3.

These data showed that the main cell metabolic processes affected by R450 were protein synthesis, amino acid metabolism, detoxification and stress response pathways, consistently with our previous proteomic analysis carried out at the same R450 dose exposure (Poirier et al., 2017). Another metabolic process proved to be affected here: the secondary metabolism especially through mycotoxin (sterigmatocystin/aflatoxin and penicillin) biosynthesis (Fig. 3 and Table 1). Maps for the top 9 KEGG pathways affected by the exposure to R450 are described in Supplemental Fig. S1.

We also compared the alteration in gene expression caused by R450 in this study to the alterations detected at the protein level in our previous investigation (Poirier et al., 2017). A total of 62 genes had their expression altered both at the transcript and the protein level (Supplemental Table S2 and Supplemental Fig. S2). Their fold changes were significantly correlated ($R2 = 0.29$, $p = 0.02$) although a large number of genes had their expression altered (up or down) in a different way at the protein level. This is not surprising since post-transcriptional processes are known to play a critical role in regulating the protein level in Aspergillus spp, as shown in other studies comparing changes in protein profiles with corresponding transcript levels (Bai et al., 2015).

3.2. Roundup stimulates ribosomal functions

The most enriched biological process annotation was translation (GO:0006412, FE = 3.0; p = 1.2E-14) (Fig. 3A). This is mainly due to the up-regulation of the expression of genes coding for ribosome components. A total of 57 genes associated to ribosome annotation (ani03010) were over-expressed (Fig. 3B). Individual fold changes were moderate and ranged from 1.50 to 2.03. They were comparable to fold changes observed in proteomic analyses for some translation elongation factors and amino-acyl t-RNA synthetases (Poirier et al., 2017). A heatmap of the ribosome-associated genes whose expression was modulated under R450 treatment displayed an unambiguous distinction between the two groups of cultures, untreated ("control") and R450-treated ("Roundup"), with a small intragroup variation (Fig. 4). Consistently with our previous proteomic analysis (Poirier et al., 2017), these results suggest that *A. nidulans* exposure to the diluted preparation of the herbicide resulted in a significant protein synthesis enhancement. This reflects a mode of action of GBH on protein synthesis in *A. nidulans* which is quite different from what is observed in plants. In plant cells, GLY inhibits EPSPS, causing a blockage of essential aromatic amino acids production and the subsequent stopping of protein synthesis (Duke et al., 2003). The shikimate pathway also exists in bacteria and fungi including *A. nidulans*. However, contrary to plant EPSPS which are GLY-sensitive (class I), some fungal and bacterial EPSPS are GLY-tolerant (class II). Whether *A. nidulans* EPSPS belongs to class I or class II remains unclear, but according to our previous data (Nicolas et al., 2016), it is not inhibited (or only partially) by GLY, suggesting that multiple cellular effects of R450 in this fungus are likely due to various targets of GLY and/or co-formulants present in the commercial mixture. The greater toxicity of the formulation when compared to GLY alone, independently of the exposed organism (including *A. nidulans*), indicates that the additives are not

inert (Braconi et al., 2006; Clair et al., 2012; Cuhra et al., 2013; Lipok et al., 2010; Mesnage et al., 2014; Mottier et al., 2013; Nicolas et al., 2016; Piola et al., 2013; Qiu et al., 2013).

3.3. Disturbance of secondary and amino acid metabolisms

One of the most significant effects of R450 exposure was a modulation of mycotoxins metabolism. Our microarray analysis revealed a down-regulation of genes involved in sterigmatocystin/aflatoxin biosynthesis (Fig. 3A, 3B and 5A) and an up-regulation of genes associated with penicillin and cephalosporin biosynthesis (Fig. 3A and 5B). Another striking effect of R450 was that it affected the metabolism of amino acids, especially tyrosine, phenylalanine, alanine, tryptophan, arginine, proline and lysine (Fig. 3B and Supplemental Table S3).

In *A. nidulans*, lysine metabolism and penicillin biosynthesis are closely interwoven (Busch et al., 2003) since penicillin is synthesized from alpha-aminoadipate, a key intermediate in lysine biosynthesis and degradation pathways (Fig. 5B). L-lysine has been shown to repress the expression of penicillin biosynthesis genes (Brakhage and Turner, 1992). In this study, the expression of several genes (ANID_00016, ANID_02545, ANID_ 03496, ANID_06961, ANID_08105, ANID_08412 and ANID_09129), identified as putative orthologs of the yeast *S. cererevisiae LYS2* gene encoding alpha aminoadipate reductase, proved to be down-regulated under R450 treatment (Supplemental Table S2). This suggests an accumulation of alpha-aminoadipate, consistent with a stimulation of the penicillin biosynthesis pathway.

A differential expression of a variety of *A. nidulans* genes related mainly to secondary metabolism, stress signalling and amino acid metabolism was already reported in the context of transcriptomic analysis of G-protein coupled receptors (GPCR) mutants compared to the wild-type strain (de Souza et al, 2013). GPCR are known to possibly act as mediators of toxic compound effects, either directly or indirectly. The direct way occurs by aberrant ligand-induced GPCR

activation resulting in the disruption of various cellular processes (Eriksson and Nordberg, 1986; Thomas and Dong, 2006; Clark et al., 2012; Jayasinghe and Volz, 2012; Fitzgerald et al., 2015). The indirect way corresponds to induction of detoxifying processes that protect the host cells from collateral damage (such as oxidative stress) caused by their defence response (Miller et al., 2015). Altogether, these data suggest an intimate relationship between stress signalling and regulation of secondary and amino acid metabolisms, consistently with results described in this report.

Secondary metabolism has been shown to be modulated in response to oxidative stress in filamentous fungi (Lee et al., 2005; Aguirre et al., 2006; Fountain et al., 2016). Many recent studies have reported the ability of GLY or GBH to induce oxidative stress (Bali et al., 2019; Burchfield et al., 2019; de Aguiar et al., 2016; de Melo Tarouco et al., 2016; Gomes and Juneau, 2016; Gomes et al., 2017; Martini et al., 2016; Mesnage et al., 2017; Murussi et al., 2016; Salvio et al., 2016; Soares et al., 2019; Wu et al., 2016), likely because GLY acts as a protonophore increasing mitochondrial membrane permeability to protons and Ca^{2+} (Olorunsogo, 1990), the latter being one of the major stimulators of the mitochondrial reactive oxygen species (ROS) accumulation (Kowaltowski and Vercesi, 1999; de Liz Oliveira Cavalli et al., 2013). Surprisingly, while the production of aflatoxin by *Aspergillus flavus* was stimulated in response to ROS exposure (Fountain et al., 2016), we observed a down-regulation of this mycotoxin biosynthesis pathway in *A. nidulans* under R450 treatment. In other studies, benzoate and amino benzoate have been shown to reduce aflatoxin production (Chipley and Uraih, 1980). Consistently, while benzoate degradation was up-regulated under ROS exposure in *A. flavus* (Fountain et al., 2016), it was down-regulated in *A. nidulans* under R450 treatment (Supplemental Table S2). However, Reverberi et al. (2005) showed that antioxidant enzymes stimulation inhibits aflatoxin production in *Aspergillus parasiticus* (incubated in the presence of lyophilised filtrates from different *Lentina edodes* isolates). Among the *A. parasiticus* antioxidant enzymes stimulated by *L. edodes* lyophilised filtrates was catalase. Some of the *A. nidulans* catalases also proved to be up-regulated under 0.007% R450 treatment (Poirier et al., 2017; this study: see

below). On the other hand, catalase was not part of the antioxidant enzymes stimulated in *A. flavus* exposed to ROS (Fountain et al., 2016). Overall, aflatoxin biosynthesis might be associated to the antioxidant status in the fungal cell, which can be modulated by external oxidative stimulus.

Mycotoxin production has been recently shown to be co-regulated with cell wall biosynthesis in *A. nidulans* (Feng et al., 2017), suggesting that a modulation of secondary metabolism regulation would also result in a modification of cell wall composition. We previously demonstrated that exposure of *A. nidulans* to R450 (at doses higher than the NOAEL one) resulted in a slight increase of the spore diameter and the hyphae width, as well as in a disruption of hyphal polarity, suggesting a modification of the wall structure affecting osmoregulation. This was consistent with proteome changes in *A. nidulans* exposed to 0.007% R450 which revealed a possible glycerol accumulation (Poirier et al., 2017). Such an increase in glycerol production has been shown to be the main response to a hyperosmotic stress (Blomberg and Adler, 1989; Nevoigt and Stahl, 1997; Redkar et al., 1995).

3.4. Detoxification pathways and stress response

Another major cluster of functional disturbances included genes involved in the response to toxic substances and cellular detoxification pathways. The annotation reflecting a response to stress (Fig. 3A) was highly enriched (GO:0006950, FE = 3.0, p = 0.001). Out of the 11 genes composing this cluster, 7 were homologs to the yeast *S. cerevisiae RTA1* gene involved in the resistance to 7-aminocholesterol, a strong inhibitor of yeast and Gram+ bacteria proliferation (Soustre et al., 1996). *RTA1* expression proved to be under the control of pleiotropic drug resistance transcription factors, suggesting a more general role in cell resistance to xenobiotics exposure (Manente and Ghislain, 2009; Kolaczkowska et al., 2012). Three other functional groups of genes involved in xenobiotic detoxification had their expression altered: cytochrome P450 (P450s) enzymes-, glutathione-S-

transferases (GSTs)-, and ATP-Binding Cassette (ABC) transporters-encoding genes. P450s and GSTs play a central role in xenobiotic metabolism, which includes 3 phases leading to the elimination of these foreign substances (Guéguen et al., 2006). While the phase I involves P450s enzymes which catalyze oxidation-reduction reactions, GSTs are part of the phase II and act by catalyzing the conjugation of reduced glutathione, via its thiol group, to electrophilic moieties of xenobiotic substrates. Phase III then consists of export of the resulting conjugated derivatives through the membranes using ABC transporters.

Cytochrome P450 genes expression was markedly altered, as indicated by an heatmap (Fig. 6) which displayed a clear segregation between the two groups of cultures, untreated ("control") and R450-treated ("Roundup) with a small intragroup variation. Genes associated with the corresponding InterPro annotation ("IPR001128:Cytochrome P450") displayed large fold-changes. Of 31 genes in this functional group, 18 were down-regulated (with a maximum fold change of -9.4) and 13 were up-regulated (with a maximum fold change of 5.5) (Supplemental Table S3). Most of these genes belonged to the CYPome (Cytochrome P450 complement) as identified by Kelly et al. (2009). Two of them (ANID_06320 and ANID_05028: *ppoB* and *ppoC*, respectively) encoded fatty acid oxygenases, which have been shown to contain a cytochrome P450 heme thiolate domain in the C-terminal region (Tsitsigiannis et al., 2005; Brodhun et al., 2010).

At least two GSTs (ANID_05831 and ANID_04905) were up-regulated with large fold changes (7 and 4, respectively) and low p-values (3.4E-12 and 1.6E-13, respectively) (Supplemental Table S3). Consistently, ANID_05831 was also found to be up-regulated (with a fold change of 3.45) in our previous proteomic analysis (Poirier et al., 2017). In *A. nidulans*, the glutathione system has been shown to interplay with the defence mechanism against hydrogen peroxide (H_2O_2) (Sato et al., 2009). Interestingly, two genes coding for putative catalases (ANID_05918 and ANID_08553), the key enzymes required to transform this harmful oxygen compound by reducing it to water, displayed a modulation of their expression under R450 treatment with a high level of statistical significance

(Supplemental Table S3). While ANID_05918 (*catC*) was up-regulated, ANID_08553 expression was decreased. Similarly, our previous proteomic data revealed that abundance of two catalases (A and B) was inversely regulated under R450 treatment (Poirier et al., 2017). Altogether, our results confirm the existence in *A. nidulans* of at least four differentially regulated catalases, giving this fungus a large capability for cellular antioxidant responses (Kawasaki and Aguirre, 2001).

The InterPro protein domain classification annotation analysis also revealed an enrichment in genes coding for characterized or putative ABC transporters (Figure 3D). They were among the most up-regulated of this study. The *atrC* gene (ANID_02349), encoding the characterized ABC transporter C (Andrade et al., 2000), was overexpressed with a fold change of 35.7, while the putative ABC transporter-encoding gene ANID_08344 had a fold change of 36.3 (Supplemental Table S3).

3.5. Chromosome mapping of genes with altered expression

In filamentous fungi, many genes involved in certain types of metabolic or developmental pathways are closely linked (Keller and Hohn, 1997). Such a cluster organization of functionally related genes suggests that some of the genes whose expression is modulated in response to an external signal would not be randomly distributed over the entire genome but localized to specific chromosomal regions. Chromosome mapping of up- and down-regulated *A. nidulans* genes under R450 treatment (FC > 1.5) indicated that 18% of them (328/1816) were closely linked to at least two others whose expression was affected in the same direction. Although many of these "bunches" of linked genes did not have a known associated function, others corresponded to well-characterized clusters, especially secondary metabolites biosynthesis gene clusters (Table 2). Such a chromosomal distribution for a significant fraction of R450-modified expression genes reflects an accurate cellular response, i.e. specific and coordinated metabolic modulations required to overcome or to offset the

effects of the herbicide. In contrast, this excludes a random transcriptional response due, for instance, to nonspecific changes in chromatin compaction along the chromosomes. However, it cannot be ruled out that the expression modulation of some closely linked genes is not due to the fact they are functionally related and belong to a specific cluster. This could be a side-effect of a very high expression modulation for one of them (high FC). Indeed, such an alteration in transcriptional expression requires, for the corresponding locus, a significant change in chromatin compaction. This important de-condensation (or condensation) of the chromatin may extend to neighbouring genes, resulting in a subsequent modification of their own transcriptional expression. Of all the "bunches" of closely linked modulated genes we identified, two (up-regulated) could fit with this hypothesis: one on chromosome I (4 genes), including ANID_06869 (FC = +121,5) encoding a putative agmatinase (Table 1; Fig. 7A), and one on chromosome VIII (4 genes), including ANID_09344 (FC = +57,5) encoding an ABC multidrug transporter (Table 1; Fig. 7B). Such a hypothesis of chromatin de-condensation side-effect, causing an up-regulation of the three 3 neighbouring genes, is supported by a fold change gradient all along the group of genes (Fig. 7A and 7B).

4. Conclusions

In this study, we performed a transcriptomic analysis of the soil filamentous fungus *A. nidulans,* using a low-density array that covers 100% of the targeted coding sequences (Deloménie et al., 2016), in order to determine the possible molecular and cellular disturbances resulting from an exposure to a commercial formulation of GBH at a dose causing no macroscopic effect. Consistent with our previous proteomic analysis carried out in the same conditions, we evidenced that this GBH (R450) affects protein synthesis, amino acid metabolism, stress response and detoxification pathways, but also secondary metabolism.

Modulation of translation genes suggested a stimulation of protein synthesis. These results confirm that the cellular and metabolic effects — and thus the lode of action — of Roundup in *A. nidulans* are totally different from those observed in plants or other organisms with a Class I EPSPS enzyme, whose inhibition by glyphosate blocks essential aromatic amino acid biosynthesis. Such a multiplicity of molecular mechanisms involved in Roundup toxicity, depending on the organism or cell type, has been previously reported (Nicolas et al., 2016). This can be explained by various targets of glyphosate or/and co-formulants present in the commercial GBH formulations which are erroneously presumed to be inert (Mesnage et al., 2015; Nicolas et al., 2016).

The up-regulation of genes involved in stress response and detoxification pathways following R450 exposure is consistent with xenobiotics elimination, but also necessary to maintain the redox balance of the cell given the well-known oxidative stress induced by GBH. Similarly, the modulation of secondary metabolism regulation, which proves to be intimately related to that of amino acid metabolism, is also part of the response to oxidative stress, the antioxidant functions of certain secondary metabolites offering an additional mechanism to handle ROS in filamentous fungi (Aguirre et al., 2006).

In conclusion, this transcriptomic analysis confirmed the multiple molecular and metabolic effects of the commercial GBH Roundup GT Plus in *A. nidulans*, despite the fact that the dose used was sufficiently low to prevent any growth and morphology disturbance. As a consequence, for any organism exposed to herbicides, the absence of visible toxic effects cannot be interpreted as a lack of metabolic alterations. This has direct implications for herbicide-tolerant genetically-modified (GM) plants which have their assessment based on the principle of substantial equivalence (i.e. close nutritional and compositional similarity between two crop-derived foods) (Aumaitre et al., 2002). A major gap is that this principle does not take into account the presence and effects of possible pesticide residues that are accumulated in crops (Cuhra, 2015). The data reported here, although based on the model organism *A. nidulans*, suggest the need to conduct integrated “omics” (including

metabolomics) studies before it can be concluded that herbicide-tolerant GM plants and their conventional counterparts are substantially equivalent.

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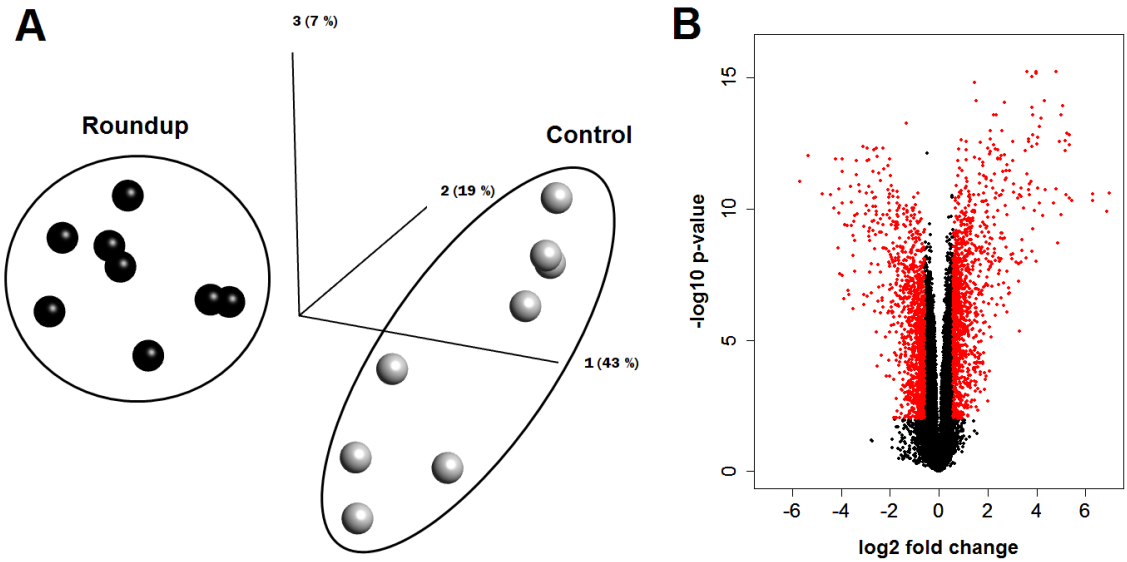

Fig. 1. Transcriptome profile alterations in Roundup-treated *Aspergillus nidulans*. Fungus grown in the presence of 0,007% Roundup (~31 mg/L glyphosate among adjuvants) for 15 h was subjected to a full transcriptome microarray analysis. **A**. Principal Component Analysis of the gene expression profiles shows a separation of treated (black) and control (grey) samples: First component (1) explains 43% of data dispersion. **B.** Volcano plots of the transcriptome profile showing the $\log_2$ fold changes and the $-\log_{10}$ p-values in gene expression induced by Roundup exposure compared to controls. Data were selected at the cut off value $p < 0.01$ and fold change > 1.5 (red).

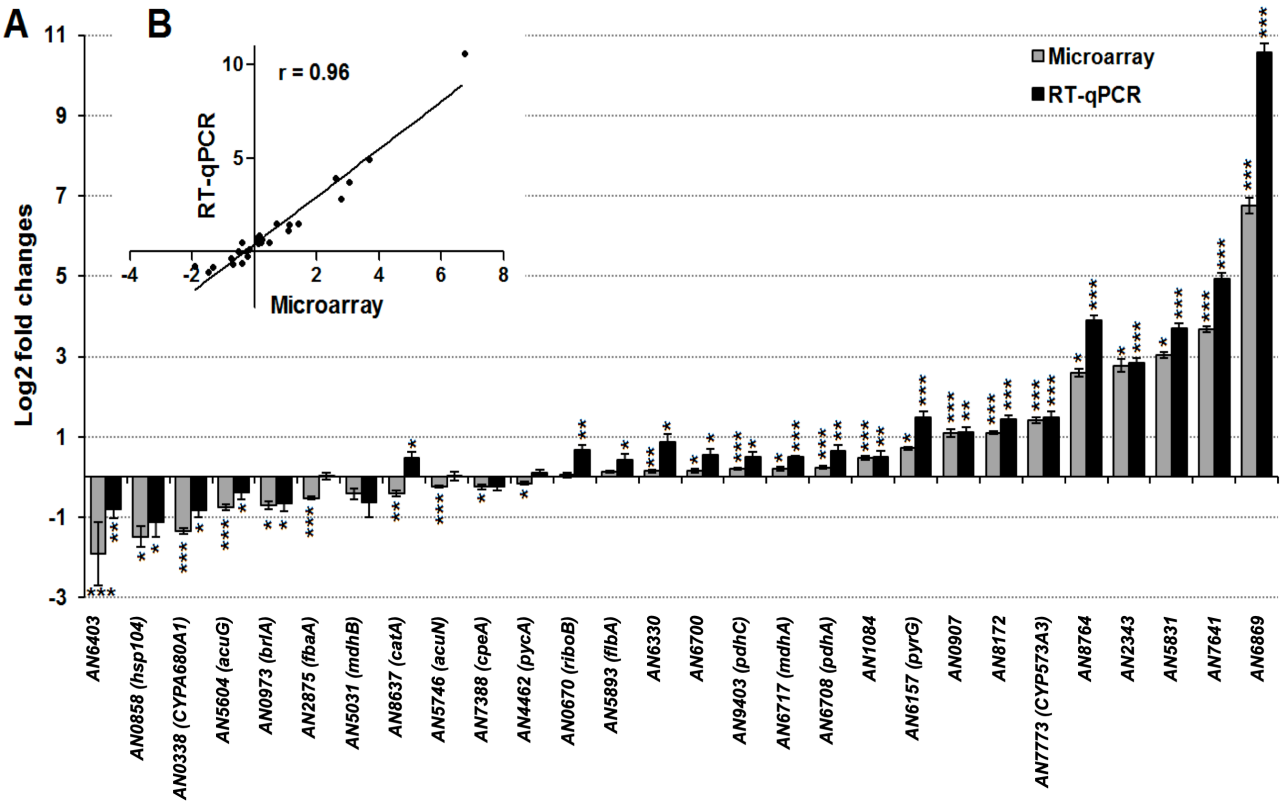

Fig. 2. Microarray data is confirmed by RT-qPCR analysis. A total of 28 genes were chosen for validation by RT-qPCR. **A.** The RT-qPCR was performed by SYBR Green I assay in duplicate and standardized against 2 reference genes (*actA* and *gpdA*). Two-tailed Student's t-tests were performed to compare the R450-treated groups to their respective controls (* $p<0.05$, ** $p<0.01$, ***$p<0.001$). Fieller's theorem was used to calculate the 95% confidence interval (error bars) of the fold changes. The corresponding current *A. nidulans* gene name, when available, is indicated between brackets. **B.** The fold-changes measured with the microarrays are very correlated with those from the RT-qPCR validation as shown by the calculation of the Spearman's rank correlation coefficient ($r= 0.96$, $p<0.0001$).

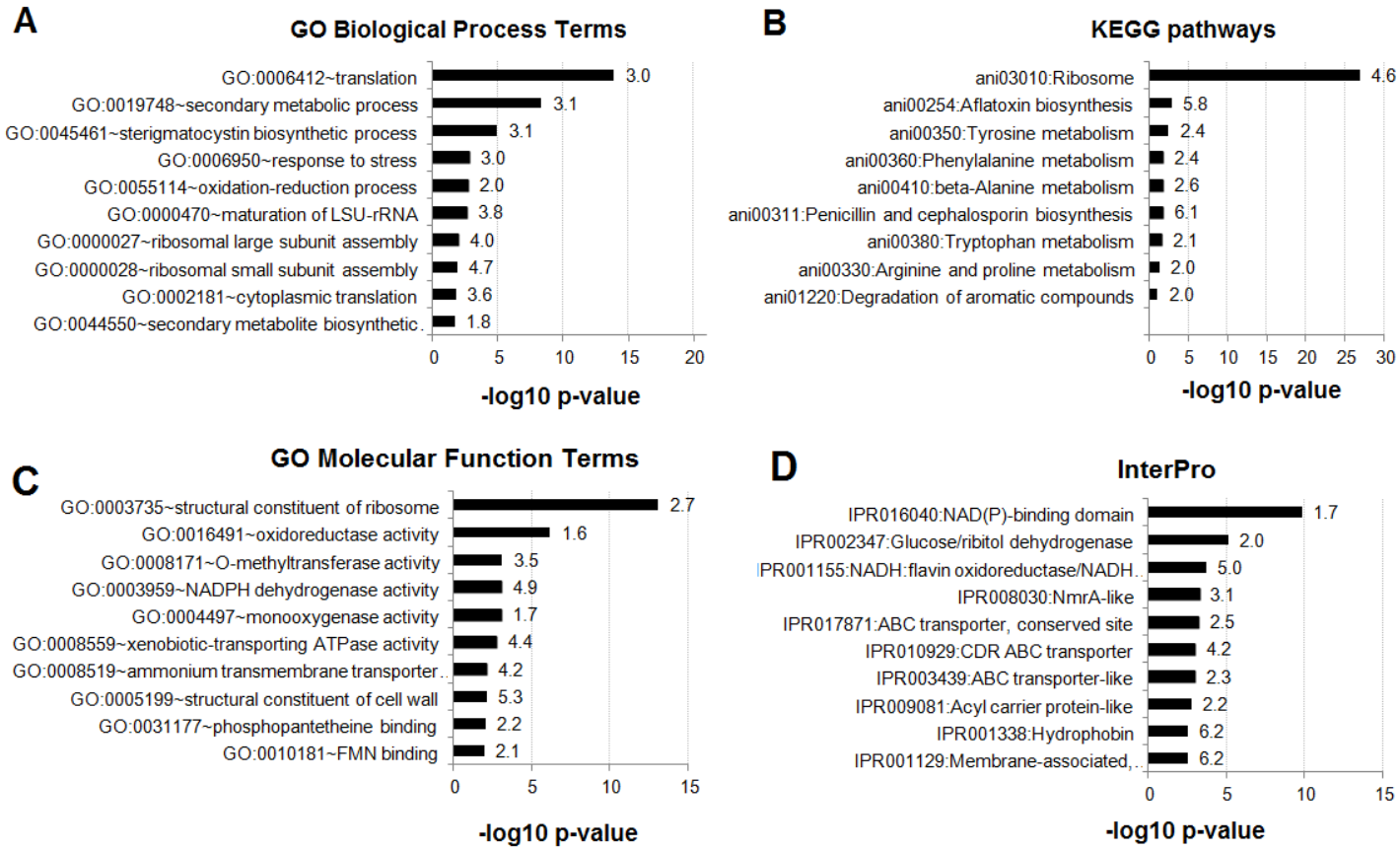

Fig. 3. Functional disturbances caused by 0.007 % R450 in *Aspergillus nidulans*. The 1,816 differentially expressed genes revealed by 2395 probes with fold-change >1,5 (p<0,01) were used to perform a Gene Ontology (GO) term **(A),** a KEGG pathway **(B),** a GO Molecular Function term **(C)**, and an InterPro **(D)** enrichment analysis. A total of 1,547 genes were recognized. The p-values calculated according to a modified Fisher's exact test (EASE score) are presented for the most enriched terms. Fold enrichments are displayed as bar labels.

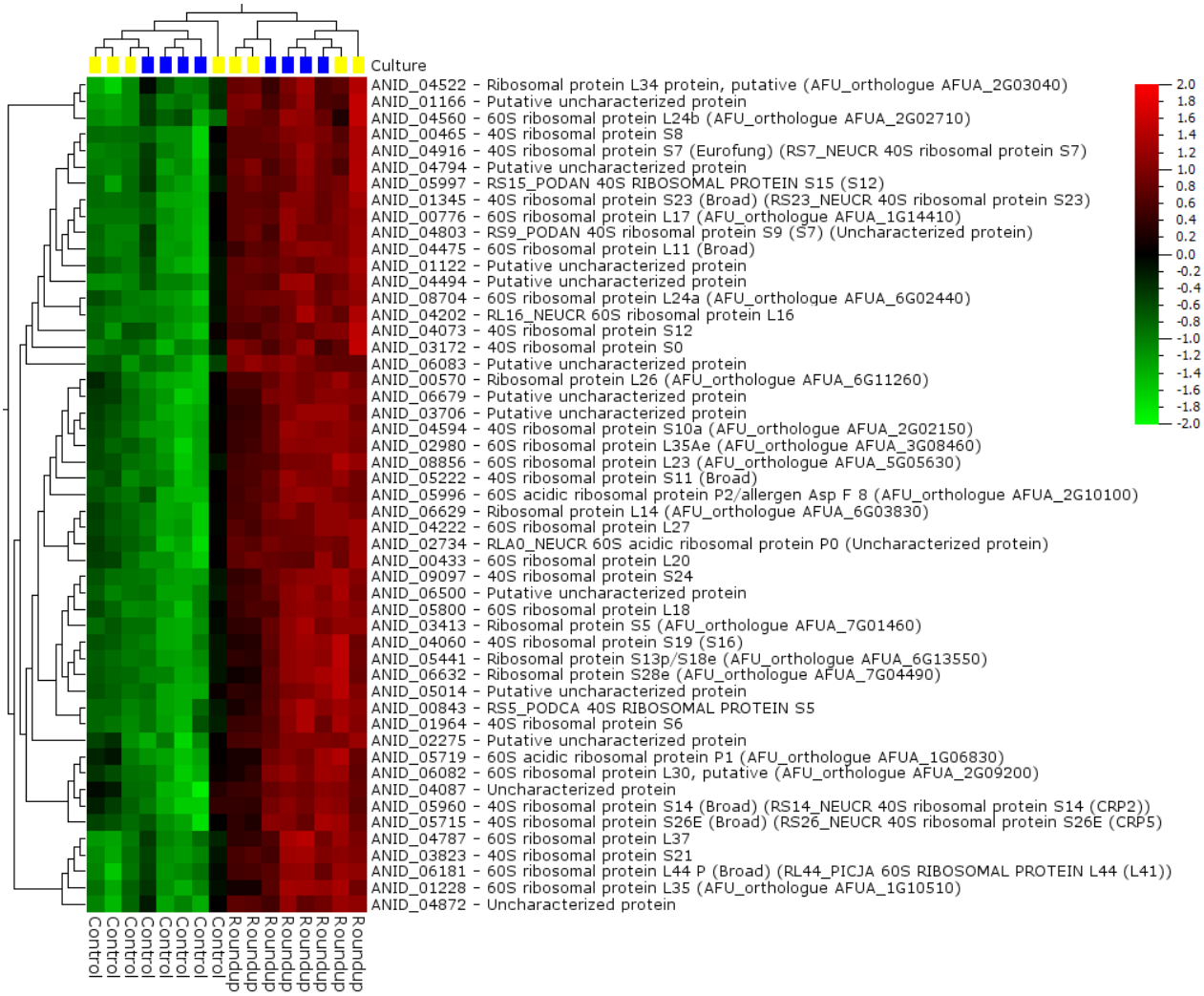

Fig. 4. Heatmaps of the major ontologically enriched ribosomal functions. Genes whose expression are displayed in this figure are part of the KEGG pathway "ani03010:Ribosome". A distinct separation based on direction (up- or down-regulation) of gene expression, between Roundup-treated and control *A. nidulans* cultures is discernible.

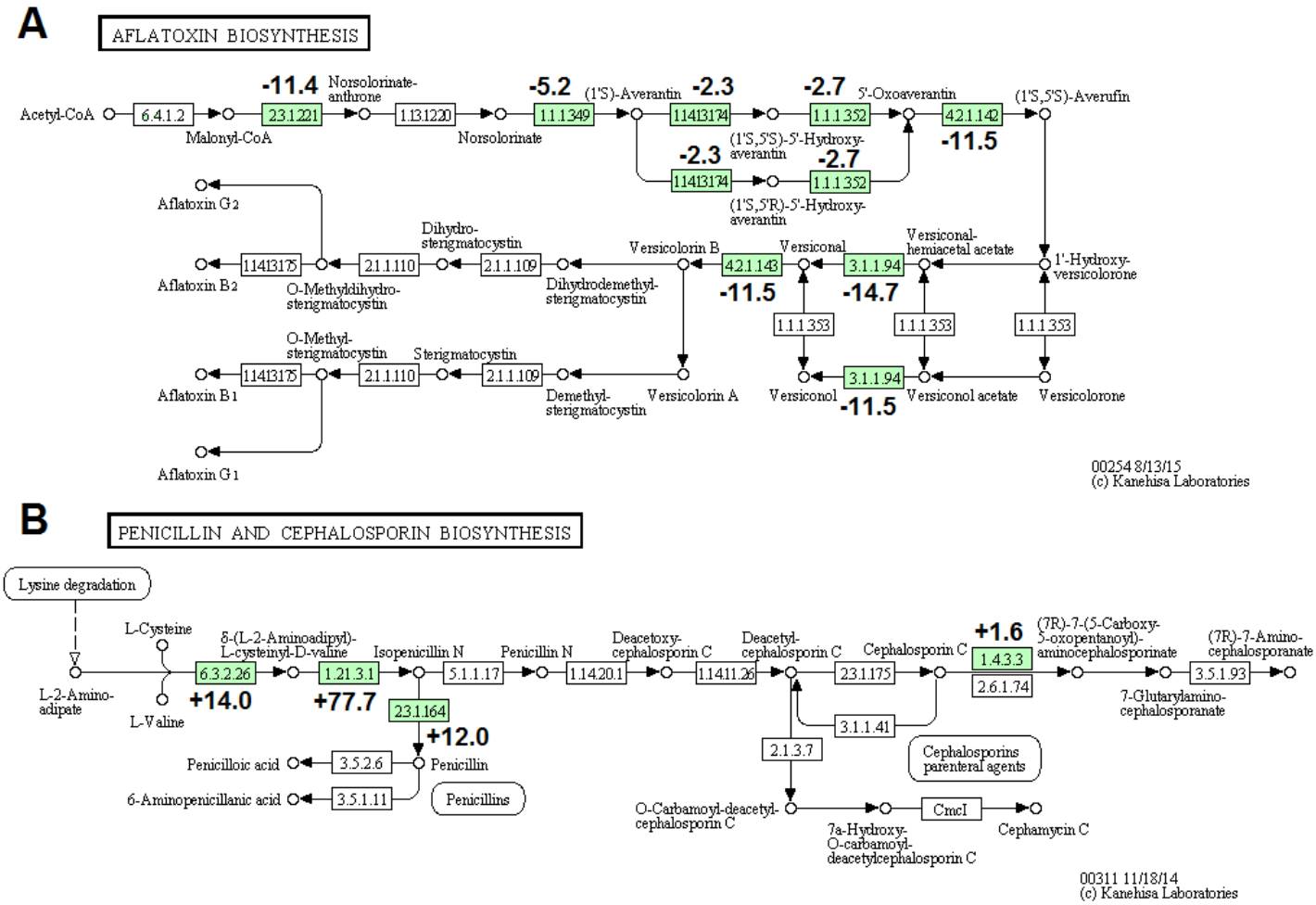

Fig. 5. Alterations of transcriptome provoked by R450 reflect a decrease in aflatoxin synthesis concomitant to an increase in antibiotic synthesis. The list of 1,816 differentially expressed genes with fold-change >1,5 ($p<0,01$) was used to represent alterations (in green) in the KEGG pathways "ani00254:Aflatoxin biosynthesis" and "ani00311:Penicillin and cephalosporin biosynthesis". Fold changes are indicated in bold characters.

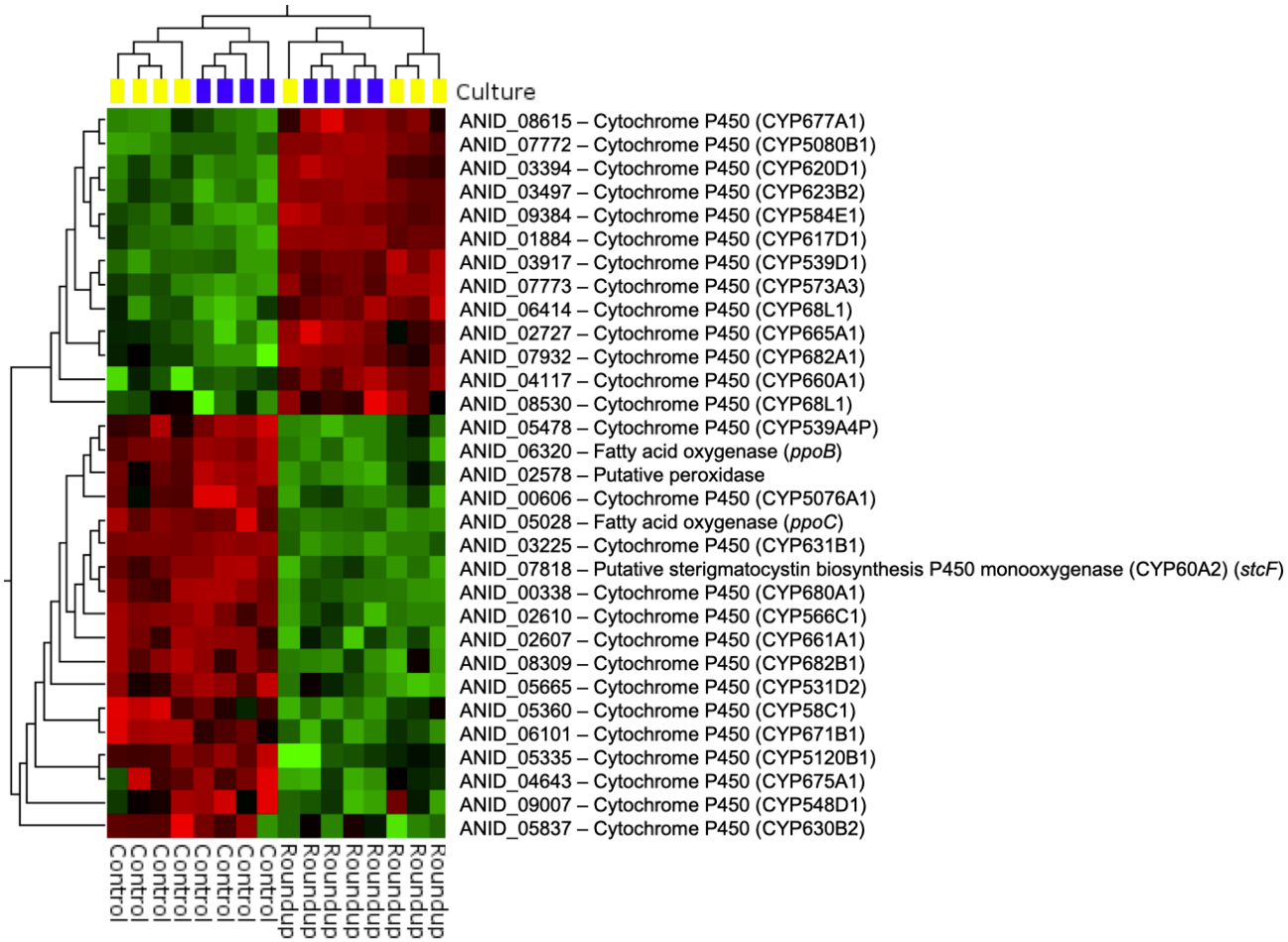

Fig. 6. GBH exposure induced changes in cytochrome P450 gene expression. The heatmap displaying differences in cytochrome P450 genes expression was made by selecting InterPro terms associated with cytochrome P450. The information given in straight letters between brackets corresponds to the *A. nidulans* P450s nomenclature as described by Kelly et al. (2009); the information given in *italics* between brackets indicates the current *A. nidulans* gene name.

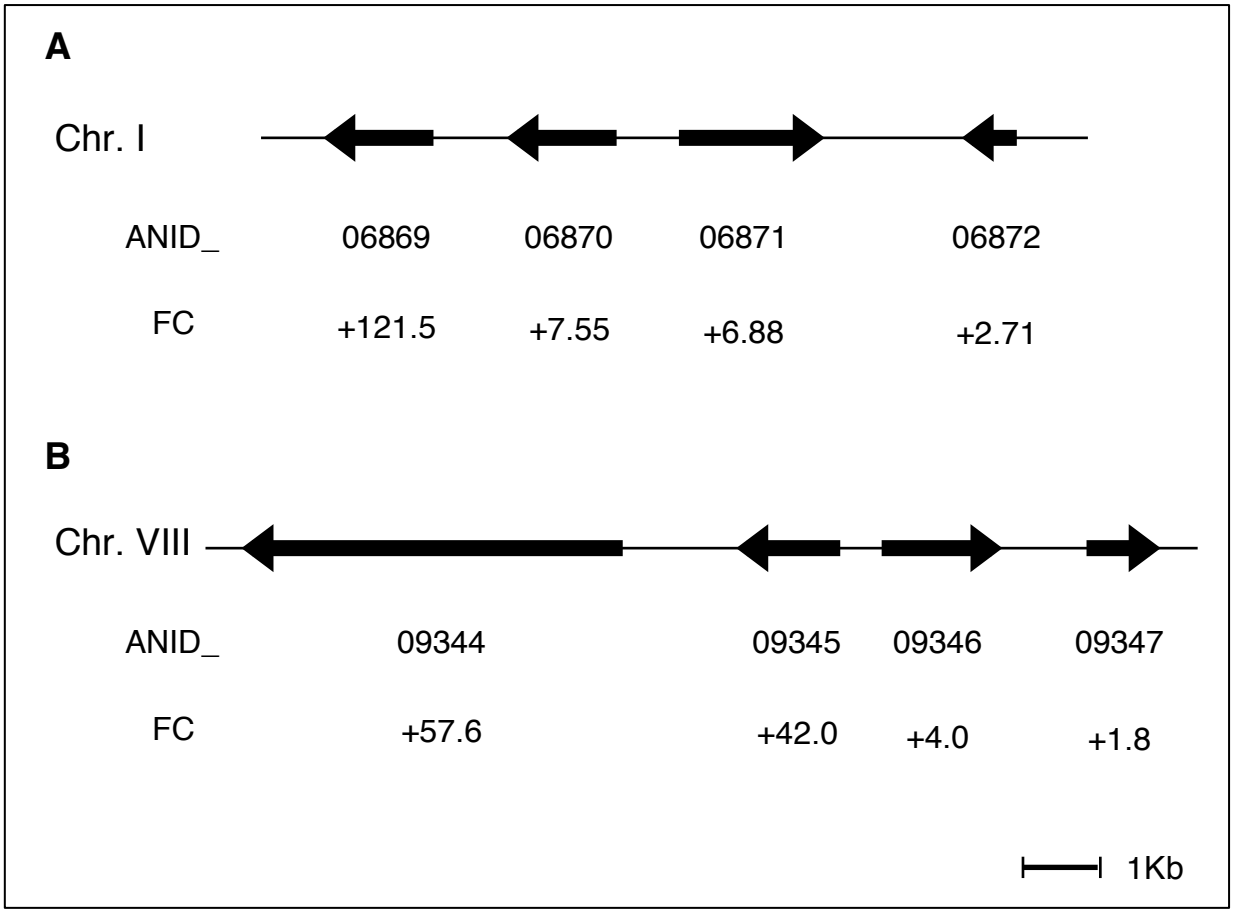

Fig. 7. Closely linked genes up-regulated by R450 treatment with a Fold Changes (FC) gradient. **A**. Group of genes located on Chromosome I. ANID with the highest FC (06869) encodes a putative agmatinase (Table 1). Metabolic functions of the three others remain unknown. **B**. Group of genes located on Chromosome VIII. ANID with the highest FC (09344) encodes an ABC multidrug transporter (Table I). Metabolic functions of the three others remain unknown.

Table 1

List of the 20 most up- or down-regulated genes after R450 exposure.

ANID[a]	Gene ID	Encoded protein (EC number)	FC[b]	p value[c]
			Up-regulated	
06869	2870566	Putative agmatinase (3.5.3.11)	+121.5	1.2E-10
02622 (*ipnA*)	2874542	Isopenicillin N-synthase (1.21.3.1)	+58.9	2.8E-11
09344	2867784	ABC multidrug transporter	+57.6	4.8E-11
09345	2867937	12-oxophytodienoate reductase (1.3.1.42)	+42.0	5.0E-11
00992	2876767	NmrA-like family protein	+40.6	1.5E-13
02349 (*atrC*)	2875142	ATP-binding cassette multidrug transport protein	+35.7	2.6E-13
00197	2875975	Uncharacterized protein	+33.6	1.2E-14
08344	2868759	ABC multidrug transporter	+33.2	5.9E-13
02561	2874590	Uncharacterized protein	+28.8	2.1E-09
07055	2870196	Cytochrome b2	+28.7	1.6E-10
08964	2868217	Uncharacterized protein	+27.8	5.8E-16
08149 (*afcA*)	2869274	Alpha-L-fucosidase A (3.2.1.51)	+25.3	1.7E-10
05467	2871758	Uncharacterized protein	+20.3	1.9E-11
05940	2870829	Putative DUF636 domain-containing protein (C-S lyase activity)	+20.1	8.0E-15
08148	2869262	Uncharacterized protein	+18.2	3.7E-14
08345	2868822	Uncharacterized protein	+17.3	7.3E-14
08963	2868228	Uncharacterized protein	+16.7	1.7E-11
07792	2869489	Putative lysophospholipase A (3.1.1.5)	+15.7	6.9E-16
02684	2873817	Putative Sterol C24(28) reductase (1.3.1.71)	+15.2	2.0E-13
03264 (*xtrB*)	2874245	Putative MFS multidrug transporter	+15.2	5.8E-10
			Down-regulated	
09273	2867822	Putative NlpC/P60-like cell-wall peptidase	-51.6	9.4E-12
05046	2872848	Anisin-1 (secreted defensin-like protein)	-41.3	9.7E-13

01567	2875642	Uncharacterized protein	-27.2	2.7E-11
01756	2875128	Uncharacterized protein	-21.6	2.8E-11
08422 (*mrvB*)	2868635	Uncharacterized protein	-19.6	9.4E-11
02538	2875721	Uncharacterized protein	-18.6	1.3E-12
07804 (*stcW*)	2869754	Monooxygenase (sterigmatocystin biosynthesis) (1.14.13.-)	-17.4	3.1E-09
08093	2868894	Putative CCCH zinc finger DNA binding protein	-17.1	1.4E-10
07816 (*stcI*)	2869364	Lipase/esterase (sterigmatocystin biosynthesis)	-15.7	2.7E-07
08611	2868559	Uncharacterized protein	-15.7	3.7E-12
08775	2868319	Uncharacterized protein	-15.7	3.4E-08
06476	2871376	Uncharacterized protein	-15.4	5.9E-11
07806 (*stcU*)	2869435	Versicolorin reductase (sterigmatocystin biosynthesis)	-13.8	4.4E-10
09142	2868118	Uncharacterized protein	-12.3	1.4E-11
07810 (*stcQ*)	2869633	Putative averufin oxydase A (sterigmatocystin biosynthesis)	-12.2	6.5E-07
07058	2869936	Uncharacterized protein	-12.0	5.7E-11
07825 (*stcA*)	2869640	Putative sterigmatocystin biosynthesis polyketide synthase (PKS)	-11.6	1.1E-09
07812 (*stcN*)	2869564	Uncharacterized protein	-11.5	6.0E-09
07809	2869531	Possibly involved in aflatoxin biosynthesis (locus in *stc* cluster)	-11.1	3.1E-10
06788	2870315	Putative Zn(II)2Cys6 transcription factor	-10.8	1.8E-10

[a]The corresponding current *A. nidulans* gene name is indicated between brackets.

[b]Fold change ; "+" or "-" indicates that the gene is up- or down-regulated, respectively. For genes whose expression alteration was revealed by several probes, the indicated FC is the average value.

[c] For genes whose expression alteration was revealed by several probes, the indicated p value is the highest one.

Table 2

Secondary metabolism gene clusters with a positive or negative co-regulation under R450 treatment

Secondary metabolite	Cluster name (reference)	Chromosome	Regulated genes[a] (up or down)
Sterigmatocystin	*stc* (Brown et al, 1996)	IV	22 (down)
Oresellinic acid/F9775	*ors* (Sanchez et al, 2010)	II	5 (up)
Aspernidine A	*pkf* (Yaegashi et al, 2013)	VI	5 (down)
Penicillin	*pcb//pen* (Montenegro et al, 1992)	VI	3 (up)
Terriquinone A	*tdi* (Bouhired et al, 2007)	V	4 (down)
Emericellamide	*eas* (Chiang et al, 2008)	VII	3 (down)

[a]Number of genes, inside the cluster, co-regulated by R450

Supplementary material

Supplementary Fig. S1. Maps for the top 9 KEGG pathways affected by the exposure to R450.

Genes having their expression affected by the exposure to R450 are indicated by red stars. **A.** KEEG Ribosome. **B.** KEEG Aflatoxin biosynthesis. **C.** KEEG Tyrosine metabolism. **D.** KEEG Phenylalanine metabolism. **E.** KEEG Alanine metabolism. **F**. KEEG Penicillin and Cephalosporin biosynthesis. **G.** KEEG Tryptophan biosynthesis. **H.** KEEG Arginine and Proline metabolism

Supplementary Fig. S2. Comparisons between fold-changes at the transcript level (this study) and at the protein level (our previous study).

We compared the fold-changes for genes having their expression statistically altered by the exposure to R450 at the transcript level (FC_transcriptomics; this study) and at the protein level (FC_proteomics; Poirier et al., 2017).

Supplementary Table S1. Primers used for RT-qPCR.

Sequences from target and reference genes were obtained from the Comparative Aspergillus Base of Broad Institute® (https://www.broadinstitute.org/). PCR primer pairs were designed with Primer3plus® (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) or PrimerBLAST (site) softwares. Oligonucleotide specificity was checked *in silico* with BLAST® (http://blast.ncbi.nlm.nih.gov/Blast.cgi) before custom synthesis by Integrated DNA Technologies (https://www.idtdna.com) or Eurofins Genomics (https://www.eurofinsgenomics.eu).

Supplementary Table S2. List of genes having their expression altered at the transcript level and the protein level.

ANID numbers (A) are used as unique identifiers. The names of the proteins (B), their fold change (C), Mascot score (D), the number of matched peptides (E), and spot numbers (F), are provided Full details are available in our previous publication (Poirier et al., 2017). These changes are compared to the changes in gene expressions for statistically significant probes (G), along with their p-values (H), fold changes (I), and detailed descriptions (J).

Supplementary Table S3. List of enriched genes found to have their levels altered in R450-treated *A. nidulans*.

Enriched genes were selected at the cut off value $p < 0.01$ and fold change > 1.5. The file contains 4 sheets. 'Data' contains the normalised intensity values for each sample and each statistically significant probe. 'Statistics' contains the results of the statistical analysis indicating fold changes (FC), as well as p-values (p) and p-values adjusted for multiple comparisons (p (Corr)). 'Variable metadata' contains gene annotations. The last sheet 'Sample_metadata' contains information on the treatment groups and the culture batch for each sample.'