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General stress response or adaptation to rapid growth in *Aspergillus nidulans*?

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ABSTRACT

Genome-wide transcriptional changes in *Aspergillus nidulans* induced by nine different stress conditions were evaluated to reveal the general environmental stress response gene set showing unidirectional expressional changes under various types of stress. Clustering the genes by their transcriptional changes was a useful technique for identifying large groups of co-regulated genes. Altogether, 1642 co-upregulated and 3916 co-downregulated genes were identified. Nevertheless, the co-regulated genes describe the difference between the transcriptomes recorded under the stress conditions tested and one chosen reference culture condition which is designated as the "unstressed" condition. Obviously, the corresponding transcriptional differences may be attributed to either the general stress response or the reference condition. Accordingly, reduced growth and increased transcription of certain antioxidative enzymes observed under stress may be interpreted as elements of the general stress response or as a feature of the "optimal growth" reference condition and decreased antioxidative protection due to "rapid growth" stress. Reversing the many to one comparison underlying the identification of co-regulated gene sets allows the same procedure to highlight changes under a single condition with respect to a set of other "background" conditions. As an example, we compared menadione treatment to our other conditions and identified downregulation of endoplasmic reticulum dependent processes and upregulation of iron-sulfur cluster assembly as well as glutathione-S-transferase genes as changes characteristic of MSB-treated cultures. Deletion of the *atfA* gene markedly altered the co-regulated gene sets primarily by changing the reference transcriptome; not by changing the stress responsiveness of genes. The functional characterization of AtfA-dependent co-regulated genes demonstrated the involvement of AtfA in the regulation of both vegetative growth and conidiogenesis in untreated cultures. Our data also suggested that the diverse effects of *atfA* gene deletion on the transcriptome under different stress conditions were the consequence of the altered transcription of several phosphorelay signal transduction system genes, including *fphA*, *nikA*, *phkA*, *srrB*, *srrC*, *sskA* and *tcsB*. Hopefully, this study will draw further attention to the importance of the proper selection of reference cultures in fungal transcriptomics studies especially when elements of specific stress responses are mapped.

1. Introduction

Environmental Stress Response (ESR) is defined as the collection of "stereotypical" transcriptional changes in a large number of genes in response to diverse forms of stress (Chen et al., 2003; Gasch et al., 2000; Gasch, 2003, 2007; Roetzer et al., 2008). ESR was first described in *Saccharomyces cerevisiae* (Gasch et al., 2000) where the authors found approximately 300 upregulated and 600 downregulated genes (co-upregulated and co-downregulated genes, respectively) under more than 20 disparate stress conditions. But this "stereotypical" behavior did not imply identical

Abbreviations: AmB, amphotericin B; AspGD, *Aspergillus* Genome Database; CR, Congo Red; ESR, environmental stress response; FC, fold change; GO term, Gene Ontology term; I, normalized signal intensity value of DNA chip experiments; MSB, menadione sodium bisulfite; PCA, principal component analysis; tBOOH, *tert*-butyl-hydroperoxide.

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kinetics and the magnitude of the transcriptional changes in ESR genes; even the identity of genes showing the highest transcriptional changes depended on the type and severity of the stress (Gasch et al., 2000; Gasch, 2003, 2007). The existence of ESR was also demonstrated in *Schizosaccharomyces pombe* (Chen et al., 2003; Gasch, 2007), *Candida glabrata* (Roetzer et al., 2008), and there are hints suggesting that ESR may also exist in *Candida albicans* (Gasch, 2007; Enjalbert et al., 2006) and in other fungi (Gasch, 2007). Most of the genes repressed in ESR can be linked to bulk protein synthesis or other growth-related processes, *e.g.* cell-cycle progression. Their repression may serve to conserve energy and mass for use during adaptation to the stress (Gasch et al., 2000; Gasch, 2003, 2007). The functions of upregulated ESR genes are more diverse; many are involved in DNA repair, oxidative stress defense, protein folding and degradation, in addition to ATP, NADPH or stress metabolite (*e.g.* glycerol, trehalose) production, or encoding proteins of different signaling pathways. Their induction may help cells maintain homeostasis under varying conditions and can be important in cross-stress adaptation processes (Causton et al., 2001; Chen et al., 2003; Gasch, 2007). The regulation of ESR genes in *S. cerevisiae* and *S. pombe* differs and depends – even within one species – on the gene of interest, as well as on the stress condition (Degols et al., 1996; Gasch, 2003, 2007; Shiozaki and Russell, 1995). In general, initiation of ESR is based on a combination of stress-specific regulators and general stress factors functioning under different stress conditions, *e.g.* Msn2/4 and Rpd3 in *S. cerevisiae* or Atf1 in *S. pombe* (Degols et al., 1996; Gasch, 2003, 2007; Shiozaki and Russell, 1995).

In a previous study, we compared genome-wide transcriptional changes in *Aspergillus nidulans* observed under different oxidative stress conditions induced by H_2O_2, *tert*-butyl-hydroperoxide (tBOOH), menadione sodium bisulfite (MSB) and diamide exposures, as well as under NaCl-elicited cationic stress (Emri et al., 2015; Orosz et al., 2017). We found that even oxidative stress responses were surprisingly diverse and we failed to detect any ESR in *A. nidulans* reminiscent of that in *S. cerevisiae*. Moreover, when comparing the behaviors of the control strain and a mutant lacking the *atfA* gene (an orthologue of *S. pombe atf1*), we found that although the numbers of co-regulated genes in the control and gene deletion mutant strains were similar, the overlap between the two co-regulated gene sets was small. This was an unexpected finding because (i) we would have expected a smaller number of co-regulated genes in the mutant if AtfA was involved in regulation of ESR, and (ii) we would have expected a large overlap between the two co-regulated gene sets if AtfA was not involved in ESR (Emri et al., 2015; Orosz et al., 2017). It is important to note that the discrepancy between our results with *A. nidulans* and those on yeasts may be due to different data processing approaches. Gasch et al. (2000) evaluated the data from a large set of DNA chip experiments with baker's yeast, and applied a clustering method to find genes showing similar expression patterns. In contrast, our transcriptomics studies with *A. nidulans* relied on a more limited number of DNA chip experiments and identified stress responsive genes based on a cut-off value applied to their transcriptional changes; we then studied the overlap among sets of stress-responsive genes from different experiments (Emri et al., 2015; Orosz et al., 2017).

Here we used the following strategy to study more thoroughly the number and function of co-regulated genes in stress-treated *A. nidulans* cultures: i) We added three new stress conditions to increase the number and diversity of studied data sets: $CdCl_2$, Congo Red (CR) and amphotericin B (AmB) exposures. ii) We compared the consequences of cut-off value-based and clustering approaches to find genes with similar expression profiles ("stereotypical" behavior). iii) We compared sets of "stereotypically" behaving genes in the control strain and a *ΔatfA* mutant to assess changes due to the deletion of the *atfA* transcription factor gene, whose functional orthologs are important in the regulation of ESR in other fungal species.

2. Materials and methods

2.1. Strains, culture conditions, stress sensitivity tests

A. nidulans TNJ 92.4 mutant (*pyrG89*, *AfupyrG*$^+$; *pyroA4*; *ΔatfA::pyroA*; *veA*$^+$) as a *ΔatfA* gene deletion strain and THS30.3 (*pyrG89*, *AfupyrG*$^+$; *pyroA*$^+$; *veA*$^+$) as the corresponding control strain were used in these experiments. The *atfA* deletion mutant was generated by double-joint PCR as previously described (Emri et al., 2015). Both strains were maintained on Barratt's (Barratt et al., 1965) nitrate minimal medium (incubation time 6 d at 37 °C). Conidia freshly harvested from these cultures were used in all further experiments.

Genome-wide transcriptional changes were recorded in submerged cultures in 500 mL flasks containing 100 mL Barratt's nitrate minimal medium broth at 37 °C and at 3.7 Hz shaking frequency. Cultures were inoculated with 100×10^6 conidia and incubated for 16 h, as previously described (Emri et al., 2015). Cultures were untreated or treated with 0.2 mM $CdCl_2$, 10 μM CR or 2 μM AmB and were further incubated for 0.5 h.

The growth inhibitory effect of the stressors was characterized by the reduction in the measured increase of dry cell mass 10 h after treatment (Emri et al., 2015). The stress sensitivities of the strains were tested on nitrate minimal medium agar plates containing 0.1–2.3 mM $CdCl_2$, 20–300 μM CR or 0.8–9.6 μM AmB. Plates were spot-inoculated with freshly made conidia suspension (5 μL, 10^5 conidia mL^{-1}) and incubated at 37 °C for 5 d (Emri et al., 2015).

2.2. Microarray analysis

Agilent 60-mer oligonucleotide high density arrays (4 × 44 K, design number 031140; Kromat Ltd., Budapest, Hungary) were used in the DNS chip studies. Total RNA obtained after the 0.5 h stress treatment was isolated from lyophilized mycelia according to Chomczynski (Emri et al., 2017). RNA samples gained from three independent experiments were pooled in a 1:1:1 ratio. Cyanine-3-labeled cRNA was prepared according to the One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling protocol (Agilent Technologies, Santa Clara, USA) as previously described (Emri et al., 2017). Fragmented cRNA samples (1650 ng; specific activity > 20.0 pmol Cyanine-3/μg cRNA) were applied to the individual arrays. Slides were hybridized at 65 °C and 10 rpm for 17 h in a rotating hybridization oven (Agilent Technologies). Slides were washed with GE Wash Buffer 1 (Agilent Technologies) at room temperature and GE Wash buffer 2 (Agilent Technologies) at 37 °C. Slides were then dried by brief centrifugation and scanned immediately on the Agilent DNA Microarray Scanner using the one color scan setting for 4 × 44 K array slides as described by Emri et al. (2015). Pre-normalized data obtained with Agilent's Feature Extraction software (version 11.1) were background corrected using the normexp + offset method (Ritchie et al., 2007) followed by quantile normalization between arrays (Smyth, 2005). The full data set (accession number GSE134562) and the old data sets (accession number GSE63019) are deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

2.3. Evaluation of the microarray data

Principal component analysis (PCA) was carried out using the normalized signal intensity data of each array with the "prcomp" function of R project (http://www.R-project.org/).

Gene set enrichment analysis was carried out with the *Aspergillus* Genome Database (AspGD) Gene Ontology Term Finder (http://www.aspergillusgenome.org/cgi-bin/GO/goTermFinder) applying default settings and biological process ontology GO terms. Only hits with a corrected p-value < 0.05 were taken into consideration during the evaluation process. Genes represented by oligomer probes on the DNA chip but modified (split, merged) or deleted from the genome during the most recent revisions (AspGD; http://www.aspergillusgenome.org), were omitted from the evaluation, and the modified gene list was used as the background gene set during these analyses.

Enrichment of the antioxidant enzyme and secondary metabolism cluster genes were tested by the Fischer's exact test with the "fisher.test" function of R project. The same "antioxidant enzyme" gene set used in the analyses was used previously (Orosz et al., 2017) and contained genes encoding known or putative antioxidant enzymes according to the AspGD gene annotations. The "secondary metabolism cluster" gene set contained manually or experimentally identified cluster genes listed by Inglis et al. (2013).

Stress-responsive genes were defined as genes displaying upregulation or downregulation in at least one stress treatment. Co-upregulated and co-downregulated genes were defined by three different methods:

1) FC (fold change) method

For $gene_i$ let $x_i = \log_2 (I_{treated,i}/I_{untreated,i})$ where I is the normalized signal intensity value obtained from the DNA chip experiments. For a fixed stress condition, $gene_i$ was considered upregulated when $x_i > FC_{cutoff}$ (where $FC_{cutoff} = 1$, unless otherwise stated); $gene_i$ was considered downregulated when $x_i < -FC_{cutoff}$; and $gene_i$ was considered stress-responsive if $|x_i| > FC_{cutoff}$. For a set of stress conditions, $gene_i$ was considered co-upregulated if it was upregulated in each stress condition and co-downregulated if it was downregulated in each stress condition.

2) D1 method

Stress-responsive gene identification (for a fixed stress condition) was based on the D1 test (twice-iterated J5 test) (Jordan et al., 2008; Patel and Lyons-Weiler, 2004). Essentially, this method is similar to the FC method but the cutoff value depends on the distribution of x_i: $D1_{cutoff} = cutoff\_scale * mean|y|$, where $y = \{x_i: |x_i| \leq cutoff\_scale * mean|x|\}$ and $cutoff\_scale = 3$ (unless otherwise stated). Similar to the FC method, for a set of stress conditions, $gene_i$ was considered co-upregulated if it was upregulated in each stress condition and co-downregulated if it was downregulated in each stress condition.

Note, since the FC and D1 methods only differ in their choice of cut-off value, the larger stress responsive gene set corresponding to the smaller cut-off value always contained the other gene set.

3) Clustering method

Genes were grouped by hierarchical clustering using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Cosine similarity ("correlation (uncentered)" setting in the software) applied on $\log_2$ FC values was used for the gene similarity metric and the average linkage method was used for clustering as described by Gasch et al. (2000). Unfortunately, the resulting clusters – with exception of very small clusters – contained both positive and negative $\log_2 FC_{gene,\ treatment}$ values. The definitions of co-upregulated or co-downregulated gene sets were, therefore, not obvious. Here, we used the following criteria: Co-upregulated (co-downregulated) gene groups were defined as the highest level clusters with at least 70 % positive (negative) $\log_2 FC_{gene,\ treatment}$ values both at their top and all direct subclusters.

3. Results

3.1. Characterization of the new (AmB, CR and $CdCl_2$) stress treatments

Unlike under oxidative stress conditions (Balázs et al., 2010; Emri et al., 2015), no significant differences were observed between the stress tolerances of the *ΔatfA* and control strains on agar plates when AmB, CR or $CdCl_2$ were selected as stressors (Supplementary Fig. 1). In submerged cultures, the employed 0.2 mM $CdCl_2$, 10 μM CR and 2 μM AmB concentrations caused 29 %, 20 % and 50 %, and 22 %, 21 % and 58 % growth reductions in the control and *ΔatfA* mutant strains, respectively.

PCA analysis of the DNA chip data demonstrated large changes in the transcriptomes under MSB, tBOOH, diamide, NaCl and AmB stress treatments, and also due to the absence of the *atfA* gene (Fig. 1). The old and new data sets showed good correlation for the untreated cultures with Pearson's correlation coefficients of 0.969 (control strain) and 0.974 (*ΔatfA* mutant).

3.2. Identification of co-regulated genes

To test whether the stress responses for *A. nidulans* showed a large overlap, co-regulated genes were identified by three different methods, as described in Section 2.3, using data obtained from all 9 stress treatments (see also Emri et al., 2015; Orosz et al., 2017).

1) FC method

The FC method yielded few co-regulated genes (Table 1). Softening the criteria of co-regulation (*i.e.* genes showing unidirectional behavior under all but one/two/*etc*. stresses) increased the number of co-regulated genes (Table 1). Alternatively, decreasing the FC_{cutoff} from 1 to 0.1 increased the number of co-regulated genes in the control and mutant strains to 223 (76 co-upregulated and 147 co-downregulated) and 267 (113 co-upregulated and 154 co-downregulated) genes, respectively, when all stress treatments were considered.

2) D1 method

The D1 method did not identify any co-regulated genes (Table 2). Relaxing the required number of unidirectional responses increased the number of co-regulated genes (Table 2). Decreasing the cut-off scale value from 3 to 0.5 (while requiring unidirectional responses in all stress conditions) increased the number of co-upregulated (co-downregulated) genes to 155 (257) and 195 (231) in the control and mutant strains, respectively.

3) Clustering method

In contrast to the previous methods based on $\log_2$ FC cut-off values, the Clustering method yielded many co-regulated genes (Fig. 2, Supplementary Table 1). However, these genes did not show strict unidirectional transcriptional changes under all stress conditions tested (Fig. 2). Among the 1642 (control strain) and 3414 (*ΔatfA* mutant) co-upregulated genes, 1000 genes showed co-regulation in both strains. In the case of co-downregulated genes, 3916 genes were detected in the control, 2530 in the mutant, and 1881 in both strains. Although the three methods (FC, D1 and Clustering methods) resulted in very different numbers of co-regulated genes, the FC and D1 methods with softened criteria

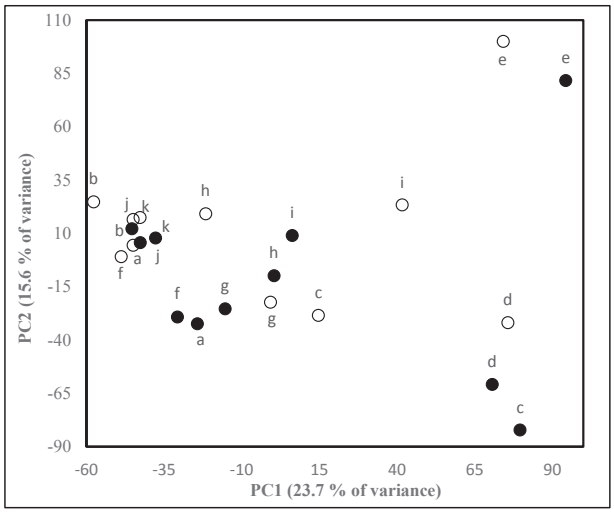

Fig. 1. PCA plot of the DNA chip data. Data obtained with the control (filled symbols) and the *ΔatfA* mutant (open symbols) strains are presented. a: untreated cultures (old; Emri et al., 2015); b: untreated cultures (new; present study); c: 0.12 mM MSB; d: 0.8 mM tBOOH; e: 1.8 mM diamide; f: 5 mM H_2O_2; g: 75 mM H_2O_2; h: 0.6 M NaCl; i: 2 μM AmB; j: 10 μM CR; k: 0.2 mM $CdCl_2$.

generally yielded genes, which were also identified with the Clustering method (Fig. 3).

3.3. Properties of the co-regulated gene sets

Decreasing the required number of treatments showing unidirectional responses allowed the identification of co-regulated gene sets containing a large number of genes and showed considerable overlap with those based on the Clustering method. We thus used the co-regulated gene sets identified with the Clustering method in all further analyses. The connection between these co-regulated genes and cellular physiology remained to be determined.

Gene set enrichment analyses detected increases in the number of genes belonging to "ribosome biogenesis", "peptide biosynthetic process"/"translation", "DNA replication" and "mitotic cell cycle" gene ontology (GO) terms in the co-downregulated gene sets for both strains (Table 3, Supplementary Table 2).

Further analyses revealed that "antioxidative enzyme" genes were enriched in the co-upregulated gene set of the control strain but not in that of the mutant strain (Supplementary Table 3). Of these, genes encoding elements of the glutathione and thioredoxine systems including *glrA* (glutathione reductase), *gpxA* (glutathione peroxidase), *trxA* (thioredoxin), *trxR* (thioredoxin reductase) and *prxA* (thioredoxin peroxidase) were notable because they were detected in the co-upregulated gene sets of both strains (Supplementary Table 3). Regarding secondary metabolism, the control strain showed enriched genes of asperfuranone (afo), terriquinone (tdi), pkb, AN11191 and AN7884 clusters in the co-upregulated gene set while genes of austinol (aus) cluster 2, and AN2924 and AN8910 clusters showed enrichment in the co-downregulated gene set (Supplementary Table 4). In the case of the *ΔatfA* mutant, genes of AN10289, AN3273 and AN8142 clusters as well as AN11194 and AN9226 overlapping clusters showed enrichment in the co-upregulated gene set while the genes of emericellamide (eas) and microperfuranone (mic), no PKS/NRPS backbone 3 and pkf clusters were enriched in the co-downregulated gene set (Supplementary Table 4).

Co-regulation of genes necessarily depends on the transcriptomes of both the reference and stress-exposed cultures and also on the overlap among stress-treated cultures. In particular, the $\log_2 FC = \log_2 I_{treated} - \log_2 I_{untreated}$ difference tends to yield $\log_2 FC$ values that decrease with increasing $\log_2 I_{untreated}$ values, resulting in genes highly expressed in the untreated culture having a higher chance of being considered down-regulated (See Supplementary Fig. 2 for an example). Consequently, the observed co-regulated sets and the biological functions of their genes reflect both the behavior of the tested strain under the untreated reference condition and features of the general stress response. The dependence of co-regulated gene sets on the reference culture also implies that selecting a different reference culture is likely to change these sets: co-regulations induced by the original reference may disappear, and new co-regulated genes may be discovered, which – at least partially – could be attributable to the new reference. For example, when we selected the transcriptome of MSB-treated cultures as a new reference, we could identify 3340 and 2649 co-upregulated genes, and 3493 and 3076 co-downregulated genes in the control and the mutant strains, respectively (Fig. 4), with a considerable overlap between them (1398 co-upregulated and 1364 co-downregulated genes). Not surprisingly, the composition of these new co-regulated gene sets differed markedly from the previous sets (Fig. 5).

In this case, the co-upregulated gene sets were enriched with "mitotic cell cycle", "DNA replication" and "translation" genes, as well as with genes belonging to the functions of the endoplasmic reticulum (*e.g.* "endoplasmic reticulum to Golgi vesicle-mediated transport", "protein localization to endoplasmic reticulum", "phospholipid biosynthetic process", "protein glycosylation to Golgi vesicle-mediated transport" genes) in both strains, while the co-downregulated gene set was rich in "iron-sulfur cluster assembly" genes in the control strain (Table 4, Supplementary Table 2).

The co-downregulated gene sets were enriched in antioxidative enzyme genes (*e.g.* elements of the glutaredoxin and thioredoxin systems, cytochrome c peroxidases and glutathione-S-transferases) in both strains (Supplementary Table 3). Regarding secondary metabolism, the control strain showed increased numbers of genes from five clusters in the co-upregulated and two clusters in the co-downregulated gene sets (Supplementary Table 4). After *atfA* gene deletion, genes from 10 clusters were enriched in the co-downregulated gene set while genes from only one cluster were enriched in the co-upregulated gene set (Supplementary Table 4).

Selecting a different strain can affect the co-regulated gene sets *via* changes in either the reference transcriptome, the stress-dependent behavior of the genes, or both (Brown et al., 2014;

Table 1
Number of genes showing co-regulation according to the FC method.

Gene set	No. of genes in the gene set	No. of genes showing stress responsiveness in								
		9	≥8	≥7	≥6	≥5	≥4	≥3	≥2	only 1
		stress(es) within the gene set								
Control strain, up-regulated genes	4033	0	1	9	51	181	474	1076	2083	1950
Control strain, down-regulated genes	4058	0	1	10	35	184	533	1170	2247	1811
ΔatfA mutant, up-regulated genes	4284	0	7	34	94	254	570	1154	2229	2055
ΔatfA mutant, down-regulated genes	3980	2	5	21	114	359	713	1330	2273	1707

Table 2
Number of genes showing co-regulation according to the D1 method.

Gene set	No. of genes in the gene set	No. of genes showing stress responsiveness in								
		9	≥8	≥7	≥6	≥5	≥4	≥3	≥2	only 1
		stresses within the gene set								
Control strain, up-regulated genes	2400	0	0	3	35	106	278	543	1084	1316
Control strain, down-regulated genes	2289	0	0	1	20	60	194	481	1123	1166
ΔatfA mutant, up-regulated genes	2455	0	3	16	51	119	247	483	1057	1398
ΔatfA mutant, down-regulated genes	1985	0	4	17	49	103	243	482	895	1090

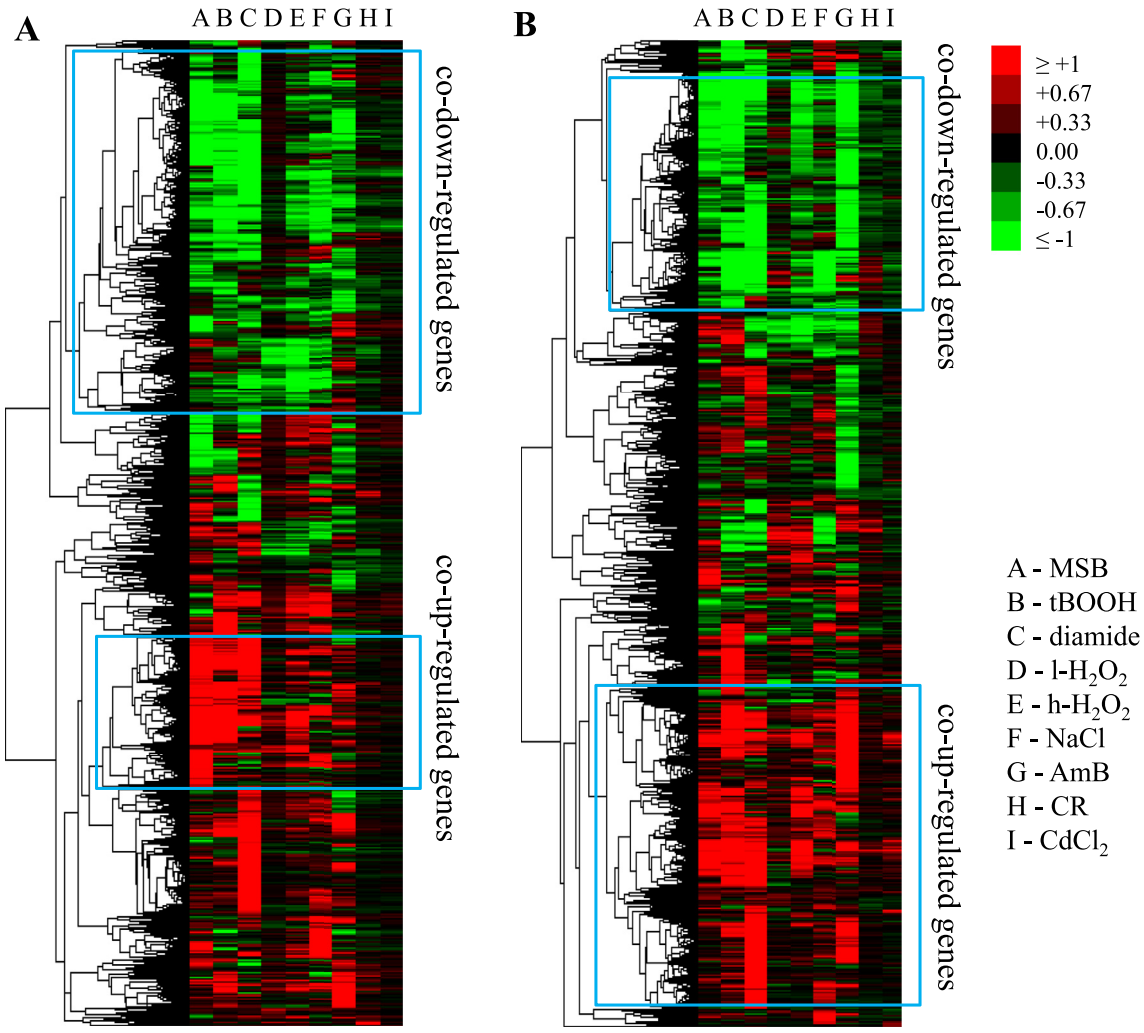

Fig. 2. Hierarchical clustering of genes based on $\log_2$ FC data, using cosine similarity, obtained with the control strain (A) or with the *ΔatfA* mutant (B). Blue rectangles mark clusters of co-regulated genes. Green colors correspond to down-regulation, red to up-regulation of a gene under a particular condition.

Kurucz et al., 2018). Here we revealed that using a *ΔatfA* mutant instead of the control strain with the same culturing conditions resulted in considerable alterations in the co-regulated gene sets (Supplementary Table 1, Figs. 2 and 4). After *atfA* gene deletion, 39 % of the co-upregulated genes (642 genes) and 52 % of the co-downregulated genes (2035 genes) lost their co-regulated nature (Fig. 6). To further investigate this loss of co-regulation due to *atfA* gene deletion, we introduced two intermediate, mixed comparisons: one comparing treated cultures of the control strain to those of untreated cultures of the mutant strain ("mutant as reference"), and one comparing treated cultures of the mutant to those of untreated cultures of the control strain ("mutant as treated") (Fig. 6). The generated gene sets are presented in Supplementary Table 1 and their relations are summarized in Fig. 6. Using the mutant as reference could account for loss of co-regulation of 1960 genes (Fig. 6 "a" + "d"), using the mutant as treated could account for 1219 genes (Fig. 6 "a" + "b"), with an overlap of 733 genes (Fig. 6 "a") accounted for by either. For 231 genes (Fig. 6 "d") loss of co-regulation occurred only when we used the mutant as both treated and reference.

Gene set enrichment analyses of the set of genes that lost co-regulation in the *ΔatfA* mutant and its four partitions created by

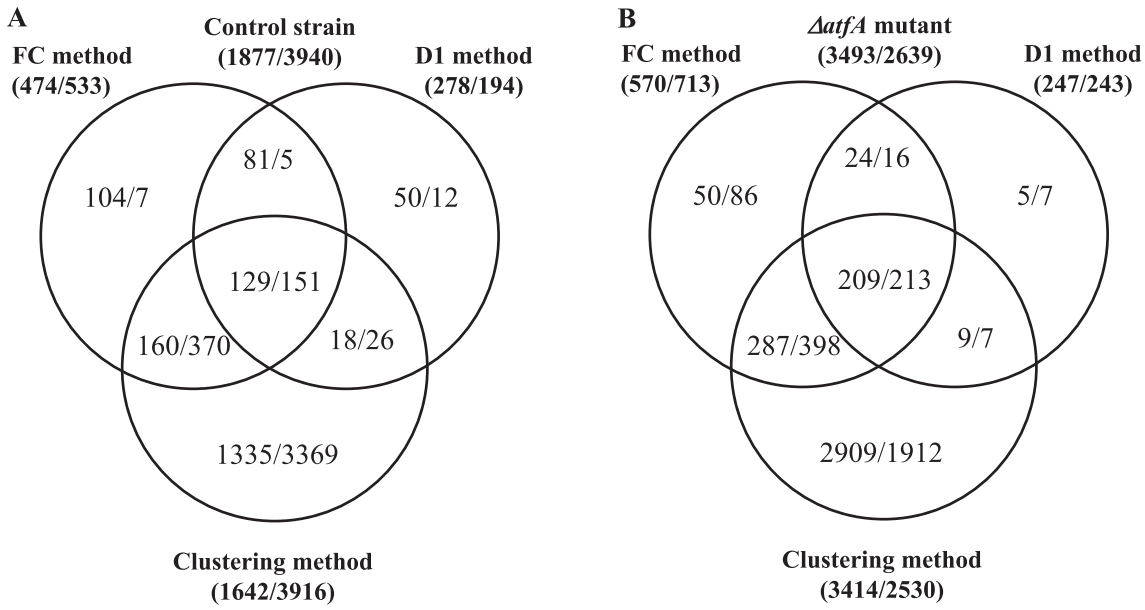

Fig. 3. Distribution of the identified co-up-regulated/co-down-regulated genes among the three methods in the control strain (A) and in the *ΔatfA* mutant (B). In case of the clustering method the original criteria (described in Section 2.3) were applied, while with the FC and D1 methods co-up-regulated and co-down-regulated genes were defined as genes showing up-regulation or down-regulation, respectively in at least 4 stress conditions (out of the studied 9).

Table 3
Selected GO terms enriched in the co-regulated gene sets generated by the Clustering method.

Gene set	Strain	Enriched GO terms[a]
co-up-regulated genes	control	iron-sulfur cluster assembly, endosome organization
co-down-regulated genes	control	hyphal growth, mitotic cell cycle, cytokinesis, DNA replication, ribosome biogenesis, peptide biosynthetic process, mitochondrial translation, lipid biosynthetic process, ergosterol biosynthetic process
co-up-regulated genes	*ΔatfA* mutant	lipid catabolic process, fatty acid beta-oxidation, peroxisomal transport, peroxisome organization, alpha-amino acid metabolic process
co-down-regulated genes	*ΔatfA* mutant	mitotic cell cycle, DNA replication, ribosome biogenesis, peptide biosynthetic process, translation, DNA repair

[a] The full dataset is available in Supplementary Table 2.

the two mixed comparisons (Fig. 6 "a"-"d") suggested that AtfA was needed not only for the regulation of normal vegetative growth (Emri et al., 2015) but also for asexual sporulation under unstressed conditions (Table 5).

To identify groups of genes that changed unidirectionally under all treatments (including untreated cultures) due to deletion of *atfA*, we applied the Clustering method to FC values calculated as $I_{mutant,i}/I_{control,i}$ where *i* is any of the eleven treatments (9 stress treatments and two untreated cultures) ("mutant/control" comparison). We found 1284 co-upregulated and 1826 co-downregulated genes (Supplementary Table 1, Fig. 7). The co-upregulated gene set was enriched with "secondary metabolite biosynthetic process" genes while the co-downregulated gene set was enriched with "phosphorelay signal transduction system" genes including *fphA*, *nikA*, *phkA*, *srrB*, *srrC*, *sskA*, *tcsB*, *hk-8-1*, *hk-8-2*, *hk-8-3*, *hk-8-4*, *hk-8-5*, *hk-8-6*, *hk-8-7* and *hk-9* (Table 6, Supplementary Table 2).

Further analyses revealed that deletion of *atfA* resulted in enrichment of genes from 10 clusters (including austinol clusters 1 and 2, derivative of benzaldehyde 1 and F9775 hybrid cluster 1, ivo cluster, and monodictyphenone cluster) in the co-upregulated gene set, while genes from only four clusters (emericellamide and sterigmatocystin clusters are notable) were enriched in the co-downregulated gene set (Supplementary Table 4). Interestingly, neither the co-upregulated nor the co-downregulated gene sets contained a significant number of antioxidative enzyme genes (Supplementary Table 3).

4. Discussion

There is no clear consensus regarding the definition of "stress"; however, stress is frequently regarded as the environmental conditions that threaten the survival of fungi (or other organisms) or at least prevent their optimal performance (Hallsworth, 2018; Hohmann and Mager, 2003). In other words, stress is a factor that forces fungi to adapt their behavior (*e.g.* through modifying their global gene expression pattern) to prevent or at least minimize reduction in their fitness (adaptive response to environmental change; Thammavongs et al., 2008). Definition of the "unstressed" condition seems to be even more problematic (Hallsworth, 2018); however, it is generally considered a unique, stress-free condition where fungi behave (grow, germinate, reproduce, *etc.*) optimally (Rangel et al., 2018) at their maximum fitness. This view seems to be inconvenient: 1) Useful and universal criteria that define this "idealistic" unstressed condition are difficult to find and reproduce in the laboratory (Hallsworth, 2018; Rangel et al., 2018). 2) Under conditions generally regarded as unstressed, fungi behaviors commonly show properties that do not correspond with the presumed behavior of this idealistic state. For example, rapid growth, which is a frequently used marker of unstressed conditions, is commonly associated with increased oxidative damage, compromised vitality and decreased competitive fitness ("rapid growth stress"; Hallsworth, 2018). 3) Most natural or industrial fungi habitats are far from "ideal" or are only close to ideal temporarily due to continuously changing parameters (*e.g.* temperature,

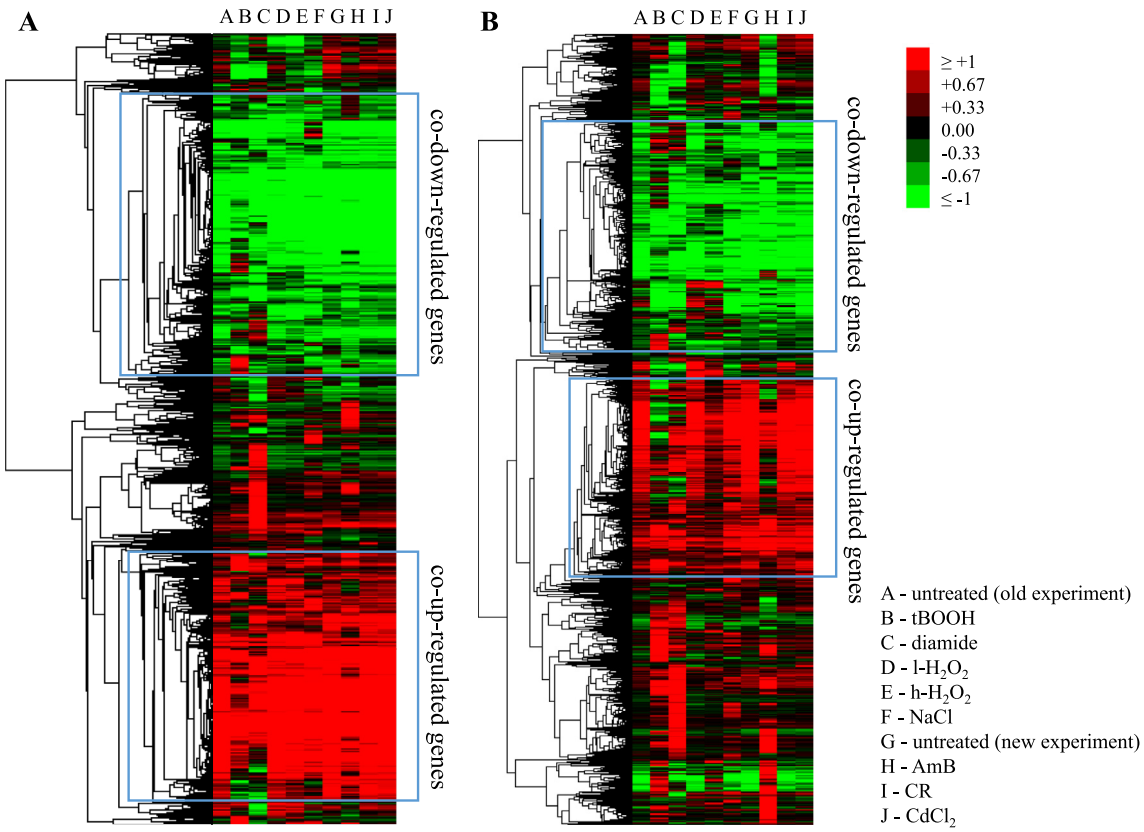

Fig. 4. Co-regulated gene sets in studies where MSB treated cultures were used as reference. (A): control strain, (B): *ΔatfA* mutant. Blue rectangles mark clusters of co-regulated genes. Green colors correspond to down-regulation, red to up-regulation of a gene under a particular condition.

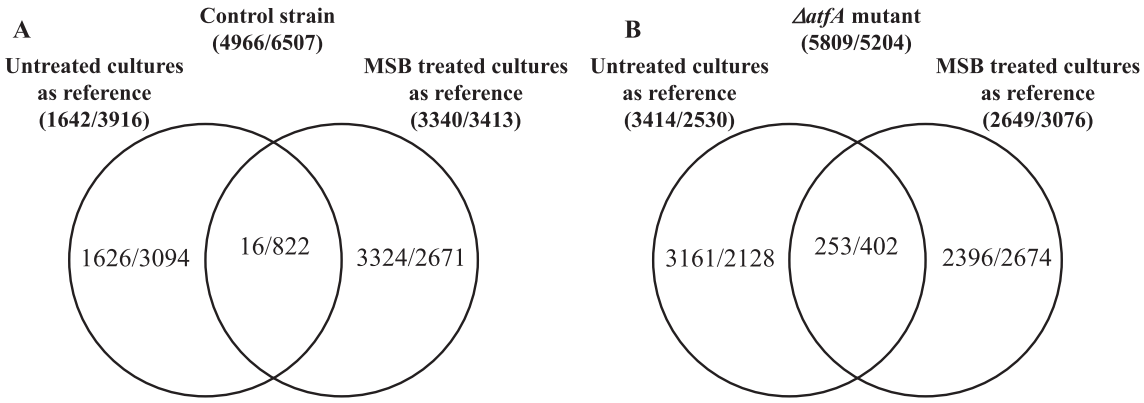

Fig. 5. Change of reference yields different co-regulated gene sets. Distribution of the co-up-regulated/co-down-regulated genes using untreated or MSB treated cultures as reference in the control strain (A) or in the *ΔatfA* mutant (B).

humidity/water activity, availability of nutrients, *etc.*). The unstressed condition would therefore be an artificially and in most cases, only theoretically, important status of cells.

Here we suggest that stress is a force that initiates adaptive changes in fungi and the stress response is the initiated change, thus the unstressed status of cells is simply a situation when nothing forces fungi to change their behavior. In other words, fungi are unstressed when they have adapted to their environment, which represents equilibrium between the microbe and its environment, rather than a special condition. Fungi can be unstressed under as many conditions as they can adapt and their growth or fitness differs in each case. For cultures adapted to a temperature, an osmotic condition or to the presence of a toxic compound, any change in the temperature, osmotic conditions or even the absence of the toxic compound can be regarded as a stressful condition if these changes initiate any adaptive response by the fungus. This modified definition of stressed/unstressed cultures can help improve our understanding of the relationship between co-regulated genes and stress responses.

Co-regulated genes can be used to describe the shared elements of different stress responses, called the general (environmental or core; Chen et al., 2003; Gasch et al., 2000) stress response. Since

Table 4
Selected GO terms enriched in the co-regulated gene sets generated by the Clustering method using MSB stressed cultures as reference.

Gene set	Strain	Enriched GO terms[a]
co-up-regulated genes	control	mitotic cell cycle, cytokinesis, DNA replication, translation, ATP synthesis coupled proton transport, Golgi vesicle transport, endoplasmic reticulum to Golgi vesicle-mediated transport, protein localization to endoplasmic reticulum, phospholipid biosynthetic process, protein glycosylation, acyl-CoA metabolic process
co-down-regulated genes	control	iron-sulfur cluster assembly
co-up-regulated genes	*ΔatfA* mutant	mitotic cell cycle, DNA replication, ribosome biogenesis, translation, inner mitochondrial membrane organization, protein targeting to ER, phospholipid biosynthetic process, carbohydrate derivative biosynthetic process, DNA repair
co-down-regulated genes	*ΔatfA* mutant	secondary metabolite biosynthetic process

[a] The full dataset is available in Supplementary Table 2.

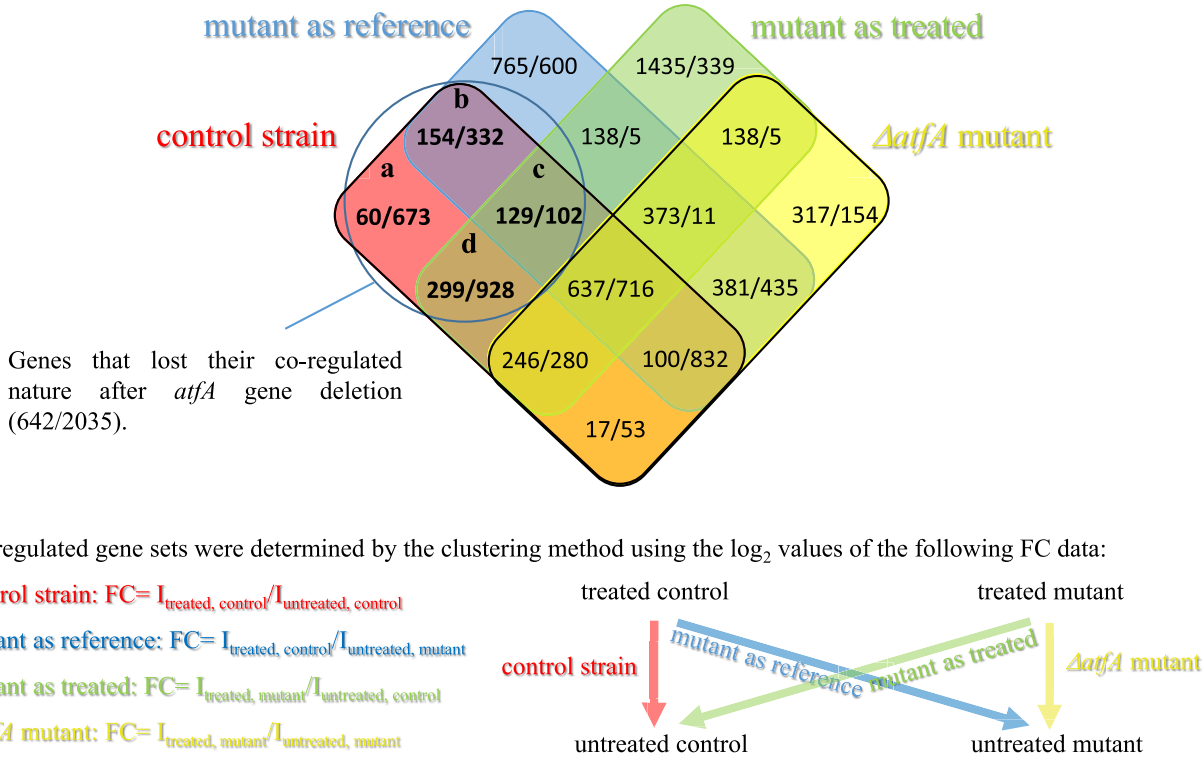

Fig. 6. Overlaps among the co-regulated (co-up-regulated/co-down-regulated) gene sets. "a"-"d": genes that lost their co-regulated nature in the *ΔatfA* mutant, partitioned according to co-regulation in the mixed comparisons "mutant as reference" and "mutant as treated". For "a" and "b", loss of co-regulation could be accounted for by using mutant as treated. For "a" and "d", loss of co-regulation could be accounted for by using mutant as reference. For "a", using mutant data on either side of the comparison could account for loss of co-regulation, for "c" loss of co-regulation only occurred in the *ΔatfA* mutant and not in either of the mixed comparisons.

Table 5
Selected GO terms enriched in the gene sets highlighted on Fig. 6.

Gene set[a,b]	Enriched GO terms[c]
co-down-regulated "total" genes	filamentous growth, generation of precursor metabolites and energy, purine-containing compound metabolic process, cellular developmental process
co-down-regulated "a" genes	purine-containing compound metabolic process
co-down-regulated "b" genes	"no significant ontology term was found"
co-down-regulated "c" genes	"no significant ontology term was found"
co-down-regulated "d" genes	filamentous growth, cell wall biogenesis, asexual sporulation, conidiophore development, conidium formation, intracellular signal transduction

[a] "total" refers to the genes that lost their co-regulated nature after *atfA* deletion. "a", "b", "c" and "d" refer to four partitions of these genes according to loss of co-regulation in "mutant as reference" and/or "mutant as treated" comparisons (see Fig. 6). "a" = loss of co-regulation in both comparisons, "b" = loss of co-regulation in "mutant as treated", "c" = loss of co-regulation in neither "mutant as reference" nor "mutant as treated", "d" = loss of co-regulation in "mutant as reference".
[b] No significant ontology term was found in case of the co-up-regulated gene sets.
[c] The full dataset is available in Supplementary Table 2.

culturing conditions can modify the reference transcriptome and the stress responsiveness of genes (Brown et al., 2014; Kaloriti et al., 2012; Kurucz et al., 2018), the general stress response is not an inherent property of the fungus but also depends on the culturing conditions. This is particularly important when working with *Aspergillus* species, which include many biomedically, industrially

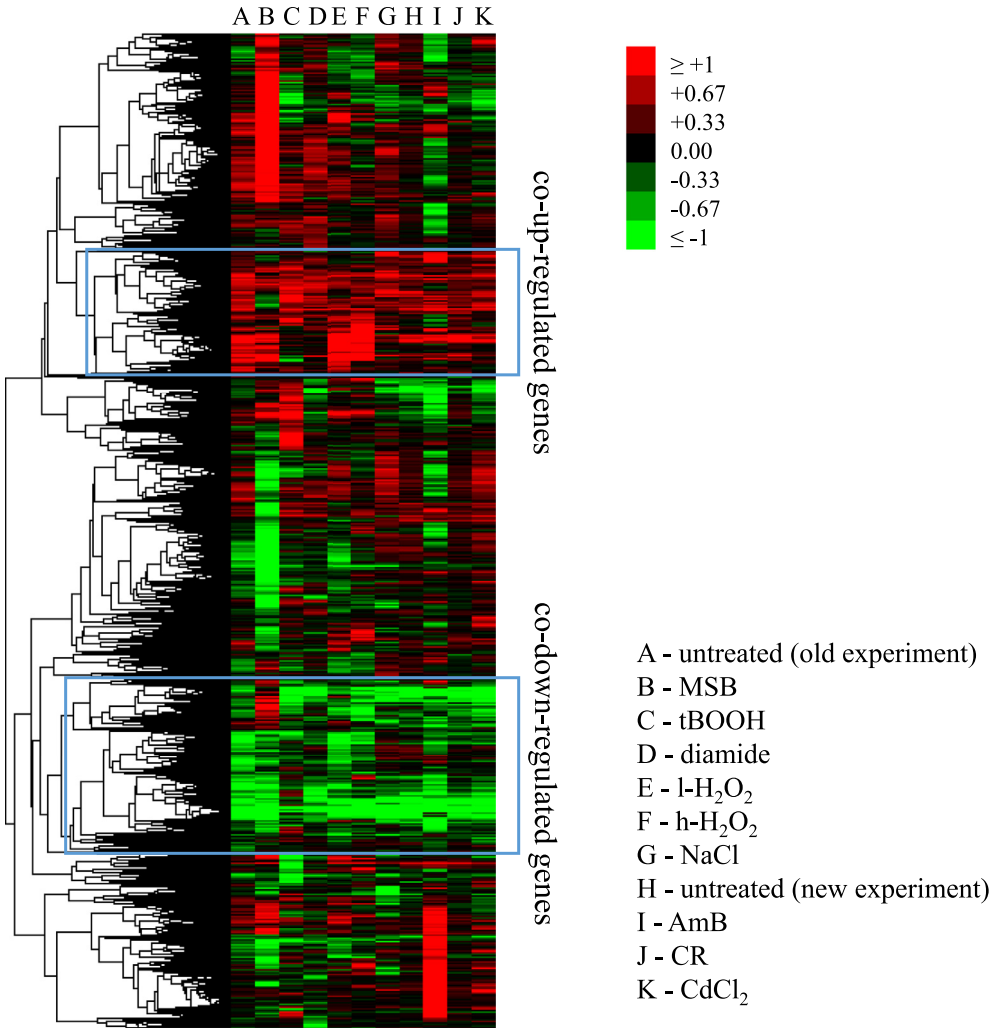

Fig. 7. Hierarchical clustering of $\log_2$ FC data, based on cosine similarity, obtained with the direct comparison of the transcriptomes of the *ΔatfA* mutant and the control strain. FC values were calculated as $I_{mutant,i}/I_{control,i}$ where i is any of the eleven treatments (9 stress treatments and the two untreated cultures) ("mutant/control" comparison). Blue rectangles mark clusters of co-regulated genes. Green colors correspond to down-regulation, red to up-regulation of a gene under a particular condition.

Table 6
Selected GO terms enriched in the co-regulated gene sets generated by the Clustering method when the transcriptomes of the mutant and the control strains were compared directly.

Gene set[a]	Enriched GO terms[b]
co-up-regulated genes	secondary metabolite biosynthetic process, austinol biosynthetic process, dehydroaustinol biosynthetic process, monodictyphenone biosynthetic process, mitochondrial respiratory chain complex IV assembly
co-down-regulated genes	phosphorelay signal transduction system, anatomical structure development, regulation of secondary metabolite biosynthetic process

[a] Co-regulated gene sets were created by clustering of $\log_2$ FC data obtained with the direct comparison of the transcriptomes of the *ΔatfA* mutant and the control strain. FC values were calculated as $I_{mutant,i}/I_{control,i}$ where i is any of the eleven treatments (9 stress treatments and the two untreated cultures) ("mutant/control").
[b] The full dataset is available in Supplementary Table 2.

and agriculturally important fungi. Standard laboratory, industrial or natural habitat conditions (including the human body in the case of human pathogens) can be very different in this case and, therefore, using different culturing conditions for different experiments is obligatory. Both the size and composition of co-regulated gene sets can vary under these diverse conditions. The general stress response in a species or strain exists only under the applied conditions and comparing the general stress responses of different species (Gasch, 2007) or strains (Fig. 2) is meaningful only if the same culturing conditions are used.

Co-regulated gene sets necessarily depend on both the reference transcriptome and all the other transcriptomes used for the analysis. The general stress response of a species mirrors similar characteristics of the reference culture. Co-regulated gene sets can be used to shed light on features of both the general stress response and adaptation to "rapid growing conditions" when "rapid growing cultures" are used as reference, as was undertaken in our experiments (Emri et al., 2015). Downregulation of growth and growth related processes (Table 3, Supplementary Table 1) due to stress occurs relative to "rapid growing cultures" and is equivalent to the

"rapid growing conditions" upregulating growth relative to the stress conditions. Similarly, when genes of a secondary metabolite gene cluster are part of the co-upregulated gene set (such as asperfuranone, terriquinone, pkb, AN11191 and AN7884 clusters for the control strain; Supplementary Table 4), this is interpreted as either stress upregulating these genes or as "rapid growing conditions" downregulating these genes. When certain types of stress enhance transcription of antioxidative enzyme genes or tolerance to another type of stress (cross adaptation), the "rapid growing conditions" presumably decreased their transcription (Supplementary Table 3) or weakened their tolerance against several stresses; thus when a treatment prevents rapid growing, cells simply return back to their "normal" stress tolerance attributes. The above-mentioned dual statements are equivalent; however, both statements should be considered when elucidating the adaptive value of the recorded changes.

Owing to the dependence of co-regulations on the reference transcriptome, co-regulated gene sets can be used to describe stress-specific changes. Co-regulated genes can be used to characterize how a reference transcriptome differs from other studied transcriptomes if a stress-treated transcriptome is selected as reference and the co-regulated gene sets are identified. In the example presented here, MSB-treated cultures were used as reference (Fig. 4), and the function of the co-regulated genes suggested that growth and growth related processes (*e.g.* mitotic cell cycle, cytokinesis, DNA replication, translation, ATP synthesis coupled proton transport) were downregulated in the MSB-treated cultures compared to the other studied stress-treated or untreated cultures (Table 4, Supplementary Table 2). Downregulation of endoplasmic reticulum dependent processes (*e.g.* endoplasmic reticulum to Golgi vesicle-mediated transport, protein localization to endoplasmic reticulum, phospholipid biosynthetic process, protein glycosylation) and upregulation of iron-sulfur cluster assembly were also characteristic of MSB-treated cultures (Table 4, Supplementary Table 2). These observations concur with our previous findings (Orosz et al., 2017) and those of other researchers who demonstrated that the endoplasmic reticulum is an important superoxide-generating cell organelle and superoxide anions can heavily disrupt Fe–S cluster proteins; therefore, downregulation of the former and upregulation of the latter processes can be important for adaptation to MSB stress, which enhances superoxide production (Perez-Gallardo et al., 2013; Popović-Bijelić et al., 2016; Rinnerthaler et al., 2012; Tan et al., 2009). Meanwhile the MSB stress-specific upregulation of glutathione-S-transferase genes (Supplementary Table 3) can be explained by the glutathione-dependent detoxification of the stressor (Castro et al., 2007).

Besides culturing conditions, strain properties can also modify co-regulations. New data concerning fungus gene mutations causing modifications to stress tolerance attributes can be collected by studying changes in co-regulated gene sets. In our case, deletion of *atfA*, which codes for a stress response regulator transcription factor (Balázs et al., 2010; Hagiwara et al., 2008; Lara-Rojas et al., 2011), substantially modified the co-regulated gene sets (Fig. 2), altering both the reference and stress-treated transcriptomes with the former having a larger contribution to loss of co-regulation (Fig. 6). This concurred with our previous findings that the involvement of AtfA in a stress response strongly depended on the applied stressor and influenced the transcription of different genes under different stresses (Emri et al., 2015; Orosz et al., 2017). According to gene set enrichment analyses (Table 5), AtfA is needed for normal vegetative growth and the normal expression of genes involved in asexual sporulation under unstressed conditions, which explains why *ΔatfA* mutants grow slower, produce less conidia than the control strains (Balázs et al., 2010; Emri et al., 2015), and may also explain, at least partly, why the conidia of *ΔatfA* mutants are stress-sensitive (Balázs et al., 2010; Hagiwara et al., 2008; Lara-Rojas et al., 2011). Interestingly, co-regulated gene sets generated after direct comparison of mutant and control strain transcriptomes (Fig. 7) included few antioxidative enzymes genes (Supplementary Table 3), indicating that regulation of these genes is complex and a missing element of the signaling network can be replaced by other elements in a stress-specific manner. Nevertheless, the presence of the *catB* gene in the co-downregulated gene set is notable since *catB* is reportedly activated by AtfA (Balázs et al., 2010). In this case, the compensatory mechanisms were not sufficient to erase the transcriptional differences caused by the missing AtfA protein. Deletion of *atfA* significantly enhanced the transcription of several secondary metabolism cluster genes (Table 6 and Supplementary Table 4) as previously reported (Emri et al., 2015), but enrichment of these genes in the co-upregulated gene set in this analysis suggests that this effect was independent of stress type. Deletion of *atfA* considerably reduced the transcription of several phosphorelay signal transduction system genes (*e.g. fphA*, *nikA*, *phkA*, *srrB*, *srrC*, *sskA*, *tcsB*) (Table 6), which explains the great number of AtfA-dependent genes (Emri et al., 2015; Orosz et al., 2017). The stress-independent effects of *atfA* gene deletion support our view that the aforementioned genes are under the direct control of AtfA.

5. Conclusions

Clustering genes by their transcriptional changes is a valuable tool to study large transcriptome sets, allowing the identification of co-regulated gene groups that are useful in characterizing common properties of stress responses. Co-regulated gene sets necessarily depend on both the transcriptome and the stress responsiveness of the genes under the reference conditions. Co-regulated genes can help describe not only the general stress response but also the characteristic properties of the chosen reference culture. Analyzing changes in co-regulated gene sets induced either by mutations or altered culturing conditions can further our understanding of these effects modifying the behavior of fungi under stress. In this study, genome-wide transcriptional changes in *A. nidulans* were induced by nine different stress conditions. Deletion of the *atfA* gene altered the composition of co-regulated gene sets substantially by altering the reference transcriptome. Future studies in this field should pay more attention to the selection of proper reference cultures depending on the specific stress responses we would like to gain a deeper insight into.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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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.2019.10.009.

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