Comparative RNA-seq based transcriptomic analysis of *Aspergillus nidulans* recombinant strains overproducing heterologous glycoside hydrolases

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ABSTRACT

Filamentous fungi are important cell factories for the production and secretion of homologous and heterologous lignocellulolytic enzymes, however the regulation of protein secretion in these organisms could be more explored. In order to investigate this regulation, *Aspergillus nidulans* recombinant strains were analyzed by transcriptomics. We designed three *A. nidulans* recombinant strains producing the heterologous proteins: alpha-arabinofuranosidase (*AbfA*), beta-glucosidase (*BglC*) and thermophilic mannanase (*1542*). The heterologous genes *abfA* and *bglC* were highly expressed, while the levels of *1542* mRNA were low and similar to a reference gene (*tubC*). There was an indirect relationship between mRNA and the protein secretion levels, suggesting that transcription is not a bottleneck for target gene expression in this system. Based on the general RNA-seq analysis of the recombinant strains, it was possible to observe that the global metabolism is up-regulated, biological processes involved on protein with binding function and cellular transport were found enriched. We also observed the unconventional splicing of *hacA* for the recombinant strains *A. nidulans*$_{\mathbf{AbfA}}$ and *A. nidulans*$_{\mathbf{1542}}$, indicating occurrence of unfolded protein response. The global analysis showed mild stress after 2 h induction of heterologous protein production, which normalized after 8 h. Our results reveal new features related to *A. nidulans* transcriptional response to heterologous proteins overproduction.

Keywords: *Aspergillus*; RNA-seq; Heterologous expression; Secretion of client proteins

INTRODUCTION

Fungi belonging to the genera *Aspergillus* features saprophytic lifestyles and have a high capacity to produce large quantities of extracellular enzymes from complex organic materials (Lowe and Howlett, 2012). The natural ability for efficient protein secretion has led to their biotechnological exploitation as cell factories for industrial enzymes production. A broad range of homologous and heterologous enzymes such as amylases, xylanases, and cellulases can be noticeably produced by *Aspergilli*, thus contributing to the expansion and growth of the enzyme market (Lubertozzi and Keasling, 2009). If continuing an annual 8.2% increase rate over the 5-year forecast period, the enzyme market is expected to reach approximately $7.1 billion by 201 8 (Reseach, 2014). *Aspergillus* is among the main enzyme-producing microorganisms, corresponding to 30% of the commercially produced enzymes, according to the Association of Manufacturers and Formulators of Enzyme Products list (AMFEP, 2015).

Despite the above-mentioned advantages, there are still many challenges to be overcome regarding the expression of target enzymes using fungal systems. It has been shown that the main bottleneck in heterologous protein production is not related to low mRNA expression (Su et al., 2012). Instead, experimental evidence suggests that targeted recombinant protein secretion in filamentous fungi is impaired due to errors in processing, modification or misfolding, hence resulting in their elimination by the endoplasmic reticulum (ER) quality control (Guillemette et al., 2007; Pakula et al., 2016). Misfolded proteins alter cell homeostasis and proper ER function, resulting in ER stress. ER stress activates conserved signaling pathways termed *unfolded protein response* (UPR) and *ER-associated protein degradation* (ERAD), which upregulate genes responsible for restoring protein folding homeostasis in the cells and degrading misfolded proteins in the cytosol by the ubiquitin-proteasome system, respectively (Heimel, 2014).

Manipulation of the UPR pathway and its components has been a common attempt to improve the production of heterologous proteins in filamentous fungi (Hayano et al., 1995; Valkonen et al., 2004; Xu et al., 2005). Many ER stress-induced UPR genes, including protein folding-related genes such as chaperones and foldases, have been co-expressed with a heterologous gene. However, the overexpression of chaperones usually does not increase the production of heterologous proteins. In *S. cerevisiae*, the overexpression of *bipA* increased the amount of extracellular prochymosin by over 20-fold, although the secretion of thaumatin was not significantly improved (Harmsen et al., 1996).

Genomic and transcriptomic approaches have been used to understand the overexpression and production of heterologous enzymes and their capacity to induce UPR, providing valuable information on *Aspergillus* genes involved with the secretion and coordination between UPR and ERAD. The induction of UPR in *Aspergillus niger* was investigated by transcriptomic analysis, comparing the UPR induced by chemicals and by overexpression of the tissue plasminogen activator

(t-PA) (Carvalho et al., 2012). Approximately 94 genes were commonly induced, most related to the functional categories protein folding, translocation/signal peptidase complex, glycosylation, vesicle trafficking, and lipid metabolism (Carvalho et al., 2012). In addition, another study showed that UPR results in activation of approximately 400 genes in *S. cerevisiae* (7-8% of the genome) (Travers et al., 2000). This reflects the complex network of interactions between UPR and other signaling pathways in the cell, mainly in relation to the secretory pathway. Transcriptome profiles can be particularly important to provide an overview of all genes and pathways regulated by UPR in the cell.

Interesting, protein improvements are non-generic, and improvements for protein A production are not necessarily transferable to protein B or C. Decades of rational and non-rational strains improvement were not available to academia due to commercial confidentiality (Meyer et al., 2016). Here, we performed a comparative transcriptome analysis of three *A. nidulans* recombinant strains producing the following heterologous proteins: 1) GH51 alpha-arabinofuranosidase (*A. nidulans*$_{\mathrm{AbfA}}$); 2) GH3 beta-glucosidase (*A. nidulans*$_{\mathrm{BglC}}$) and 3) GH5 thermophilic mannanase (*A. nidulans*$_{1542}$). These enzymes have very different domains and posttranslational modifications, so our interest was identify commonly differentially expressed (DE) genes that potentially represent a general adaptation of the cell to the overexpression of heterologous genes, including a hyperthermophilic gene. The transcriptional profiles of the *A. nidulans* recombinant strains were determined at early and late periods of induction. Furthermore, 17 genes related to the secretion pathway were DE in all the recombinant strains. Our results provided insights on understanding how *A. nidulans* adapts to the overproduction of heterologous proteins and highlighted new potential targets for genetic manipulation and improvement of the protein secretion yields in this microbial system.

RESULTS

Recombinant proteins are secreted in different levels

In order to understand the heterologous protein production in *A. nidulans*, three different proteins were monitored: GH51 alpha-arabinofuranosidase (AbfA) and GH3 beta-glucosidase (BglC) both cloned from *Aspergillus fumigatus*, and a thermophilic GH5 mannanase (1542) cloned from the hyperthermophilic bacterium *Thermotoga petrophila*. The target sequences were cloned into a pEXPYR vector and transformed into *A. nidulans*$_{\mathbf{A773}}$ (Segato et al., 2012). These recombinant strains were selected due to an increased secretion level of the target proteins, *i.e*, AbfA > BglC > 1542. The transformed strains were denominated *A. nidulans*$_{\mathrm{AbfA}}$, *A. nidulans*$_{\mathrm{BglC}}$ and *A. nidulans*$_{1542}$.

Initially, the time course of heterologous enzymes secretion was evaluated by growing the recombinant strains in maltose to induce the *glaAp* promoter (Segato et al., 2012) and activate expression of the heterologous genes (**Figure 1**). Despite the differences in secretion of the target

proteins, the three strains showed similar profiles of mycelium dry weight, amount of extracellular proteins and pH of the media. The similarity among these parameters indicates that the overexpression of heterologous genes may not imply to major physiological changes of the strains.

The AbfA presented the highest level of recombinant protein secretion among the three strains, with an activity peak at 48 h (9.53 U/ml) (**Figure 1A;** *middle panel*). The BglC secretion level was lower than AbfA and the peak activity was early at 24 h (**Figure 1B;** *middle panel*; 8.73 U/ml). In general, after 48 h of cultivation, a protease-degradation profile was observed (**Figure 1**; *bottom panel*) for the three recombinant strains. At this cultivation time, cells probably undergo disruption due to the absence of nutrients. A higher beta-glucosidase activity was detected from 72 to 120 h by using pNPG (**Figure 1B;** *middle panel*), but this activity was probably due to a native beta-glucosidase (AN2828; corresponding to an intense protein band between 75 and 85 kDa) whose amino acid sequence/identity was further confirmed by LC-MS/MS (data not shown). In contrast, the enzyme 1542 could not be observed in the gel, however some residual activity (under 0.6 U/ml) was detected in similar amounts from 18 to 120 h, suggesting that this thermophilic enzyme was resistant to degradation (**Figure 1C;** *middle panel*). It is important to emphasize that the enzymatic activity of this mannanase was measured at high temperatures (87 ºC), avoiding the possibility for false positive results, *i.e*, the activity of a native enzyme. Secretion of proteins into the medium driven by strong promoters (such as *glaAp*) is often subjected to extracellular proteolysis. This is a very common phenomenon, occurring in filamentous fungi that secrete native proteases, or in response to glucose starvation or pH increase (Segato et al., 2012).

In order to determine whether the low intensity BglC and 1542 bands were caused by problems during protein folding and/or secretion, SDS-PAGE and enzyme activity assays were carried out in the intracellular fraction (**Figure S1**). The overexpression of heterologous proteins may cause their intracellular accumulation, suggesting breakdown or overloading of the secretory pathway (Sims et al., 2005). Protein bands related to the recombinant proteins were not observed in the intracellular fraction, indicating that the target proteins did not accumulate inside the cell.

In general, we observed that the secretion levels does not affect the overall physiology of the strains. The levels of target protein secretion increased from 1542 to AbfA and an intermediate level was observed for BglC. Thus, we sought to understand at the transcriptomic level whether these differences in heterologous enzymes secretion were related to their mRNA expression.

The heterologous genes are highly expressed in *A. nidulans*

In order to investigate whether the differences in heterologous enzymes secretion were related to mRNA expression, expression of the target genes *abfA*, *bglC* and *1542* were measured by qPCR. The *abfA* and *bglC* genes were highly expressed when compared to the endogenous reference gene

(*tubC*), while the levels of *1542* mRNA were similar to the *tubC*. The genes were detected even after 72 h of maltose induction (**Figure 2A**).

The production of recombinant proteins in filamentous fungi by using strong promoters activates some regulatory genes related to protein quality control and UPR (Liu et al., 2014; Nevalainen and Peterson, 2014; Pakula et al., 2003). The UPR represents an adaptive response that aims to restore cellular homeostasis triggered by ER stress (Guillemette et al., 2011). UPR target genes such as *bipA* and *sec63* were quantified in function of time (**Figures 2B and C**) (Cerqueira et al., 2014; Heimel, 2014; Sims et al., 2005; Wood et al., 2012). The *bipA* gene was overexpressed in the three recombinant strains at 12 h and exclusively in *A. nidulans*$_{\mathbf{AbfA}}$ at 8 h (**Figure 2**), probably due to the higher AbfA production compared to the other recombinant proteins. The *sec63* gene was overexpressed in *A. nidulans*$_{\mathbf{AbfA}}$ and *A. nidulans*$_{\mathbf{1542}}$ at 8 h and in *A. nidulans*$_{\mathbf{1542}}$ only at 12 and 24 h. Late overexpression (48 and 72 h) of *bipA* and *sec63* is probably related to the absence of nutrients, considering that maltose was totally consumed (**Figure S2**), causing starvation, UPR and cell death (Szilágyi et al., 2013). In *Trichoderma reesei*, protein overproduction induced the overexpression of UPR genes only at early cultivation stages (from 1 to 12 h) (Wang et al., 2014) and, after 12 h, the quantity of UPR target returned to basal levels. Heterologous expression of the bacterial xylanase B (*xynB*) in *A. niger* also showed lower mRNA levels of some UPR target genes (*gla*, *bip*, and *hac1*) (Zhang et al., 2008). Here, we observed the overexpression of *bipA* and *sec63* primarily at 12 h after induction of the heterologous gene expression. Based on the time course of recombinant protein production, the fast maltose consumption and qPCR data, we defined 2 and 8 h of heterologous genes expression as standards to understand how the *A. nidulans* strains adapt to the production of heterologous proteins.

The indirect relationship between high mRNA levels (**Figure 2A**) and low levels of secreted proteins (**Figure 1B and C**) was also reported for cellobiohydrolases (CBHs) from *Aspergillus terreus* and *T. reesei* heterologously expressed in *Aspergillus carbonarius* (Zoglowek et al., 2015). These authors speculated that proteolytic degradation could be one reason, possibly along with other factors such as incorrect folding, post-translational processing, and impairment of intracellular transport (Zoglowek et al., 2015). The indirect relationship between mRNA and protein levels is frequently reported, suggesting that transcription is not a bottleneck in these systems. In an attempt to establish a set of cellular processes altered during the adaptation of *A. nidulans* to the overexpression of recombinant genes, we analyzed the three recombinant strains by RNA-seq at 2 and 8 h.

Global transcriptional response to heterologous protein production

Differential expression analysis was performed by pairwise comparisons of each recombinant strain and the control strain *A. nidulans*$_{\mathbf{A773}}$. We identified 476 differentially expressed (DE) genes by applying the pipeline and filters described in the Methods section (**Figure 3**). Except for the strains BglC and 1542 at 8 h, there was a global transcriptional up-regulation response during the overexpression of recombinant genes in *A. nidulans.* Thirty out of 476 DE genes were common for the three recombinant strains. *A. nidulans*$_{\mathbf{AbfA}}$ and *A. nidulans*$_{\mathbf{1542}}$ also showed 74 DE genes in common. This observation is supported by the Venn diagrams and PCA analysis (**Figure 3 and Figure S3**).

To better understand how the general cellular processes are regulated during the production of recombinant proteins in *A. nidulans*, the annotation of DE genes was performed with FunCat (Ruepp, 2004). The most significant functional category was "metabolism", followed by "protein with binding function or cofactor", "cellular transport" and "cell defense". These four categories were enriched in all recombinant strains. In a more detailed analysis of the functional subcategories (**Figure 4),** we found several altered processes that are related to cellular stress, such as the subcategories metabolism, transport routes and cellular defense. These subcategories were also reported in other *Aspergillus* species overproducing recombinant proteins (Kwon et al., 2012; Liu et al., 2014; Zhou et al., 2016). The overexpression of glucoamylase (GlaA) in *A. niger* resulted in the activation of "translocation", "protein glycosylation", "vesicle transport" and "ion homeostasis" processes (Kwon et al., 2012). The functional categories "cellular transport", "amino acid metabolism", "aminoacyl-tRNA biosynthesis" and "metabolism" were overrepresented in an *Aspergillus oryzae* strain expressing a constitutively active form of *hacA*, indicating its importance for UPR, fungal growth, and physiology (Zhou et al., 2016).

Thirty DE genes were common to all the recombinant strains and three patterns of expression were observed. Genes upregulated in 2 and 8h, genes downregulated in 2h and upregulated in 8h, and genes upregulated in 2h and downregulated in 8h (**Figure 5, Additional file 1: Table S1 and S2**). These common genes are related to general biological process such as sexual sporulation, defense, detoxification and secondary metabolism, and it is likely to represent a common response of *A. nidulans* cells to recombinant proteins production independent of protein features.

The basic sensing pathway to detect ER stress or an increase in the folding load is highly conserved from yeast to humans. The increase in misfolded proteins stimulates the Ire1 autophosphorylation and dimerization, triggering the unconventional splicing of an intron in the *hacA*^{u} to create the transcriptionally active form *hacA*^{i} (Guillemette et al., 2011). HacA is a conserved bZIP transcription factor in eukaryotic cells, regulating gene expression in response to various forms

of secretion stress and as part of secretory cell differentiation (Carvalho et al., 2012). In order to evaluate the canonical pathway of UPR, we analyzed the ratio of *hacA* splicing in the *A. nidulans* recombinant strains based on the RNAseq data (**Figure S**4). The levels of *hacA* splicing were higher for AbfA and 1542 than for BglC at 2 h, and after 8 h the levels of *hacA* splicing for all the recombinant strains were lower than the control strain, showing a normalization of the UPR canonical pathway. This result suggests either no accumulation of the BglC misfolded form or the presence of an alternative pathway of ER stress and UPR. On the other hand, for AbfA and 1542 it was possible to infer that there is misfolded protein accumulation at 2 h. Wang et al (2014) analyzed *hac1* levels in the *T. reesei* Rut C30 and QM9414 strains. The transcript levels of total *hac1* increased earlier in Rut C30 (1 h of induction) and this response was maintained longer than that in QM9414. Compared to our data, during the expression of recombinant genes, the *hacA* expression appears to be up-regulated at earlier times, with a response similar to that from *T. reesei* (Wang et al., 2014).

Based on the general analysis of RNA-seq of the recombinant strains, it was possible to observe a global up-regulated transcriptonal response. Moreover, biological processes related to metabolism, protein with binding function and cellular transport were enriched. Although there are not many processes related to cell stress, we observed the unconventional splicing of *hacA* for *A. nidulans*$_{\mathbf{AbfA}}$ and *A. nidulans*$_{\mathbf{1542}}$, indicating some level of ER stress. The global analysis showed mild stress at 2 h after inducing heterologous protein production, which was normalized after 8 h.

Differential expression of genes related to the secretion pathway

The production of heterologous proteins is not only impaired by low expression of heterologous genes, but it can also be hampered by problems in post-translational processing in the secretory pathway (Yoon et al., 2010). Many studies have attempted to comprehend the superior secretion capacity of *Aspergillus*, primarily at the transcriptomic level (Carvalho et al., 2012; Guillemette et al., 2007; Kwon et al., 2012; Liu et al., 2014; Sims et al., 2005). These studies identified important genes in different stages of protein production such as translocation, folding, cargo transport and exocytosis (Schalén et al., 2016). Liu et al. (2014) defined a set of genes involved in the secretion pathway of *A. oryzae* using the secretory model of *S. cerevisiae* as a scaffold. Based on this list, we defined the homologous or the best hit genes in *A. nidulans* by using the AspGD data, generating a set of 374 genes (**Additional file 2: Table S3**).

Seventeen genes were DE in the recombinant strains (**Additional file 2: Table S4**) and each recombinant strain showed a singular response. *A. nidulans*$_{\mathbf{1542}}$ showed a predominant down-regulation response, while *A. nidulans*$_{\mathbf{BglC}}$ had an up-regulation response (**Figure 6A)**. *A. nidulans*$_{\mathbf{AbfA}}$ also presented more genes in common with *A. nidulans*$_{\mathbf{1542}}$ than with *A. nidulans*$_{\mathbf{BglC}}$ (**Figure 6B**). In order to understand the functional categories represented by the 17 DE genes of the secretion

pathway, a functional categorization was performed using FunCat. The most represented categories were “stress response”, “protein folding and stabilization” and “unfolded protein response”, with 10, 8 and 8 genes, respectively (**Figure 6C**).

In general, the similar pattern between *A. nidulans*$_{\mathbf{AbfA}}$ and *A. nidulans*$_{\mathbf{1542}}$ was evident and the overrepresented biological processes corroborated this conclusion: “protein folding and stabilization”, “protein modification” and “stress response”. We considered some explanations for this observation, first that the enzyme BglC is a complex protein with 797 amino acids, and according to NetNGlyc 1.0 it is predicted to have five N-glycosylation sites, while AbfA and 1542 are smaller proteins (656 and 667 amino acids, respectively) with three and one predicted N-glycosylation sites, respectively. *A. nidulans*$_{\mathbf{BglC}}$ showed six exclusively up-regulated genes related to the secretion pathway, with five chaperones genes (AN2731, AN6010, AN6089, AN8269 and AN9124) involved in protein folding, stabilization, targeting, sorting and translocation (**Additional file 2: Table S4**). This observation also indicates the hypothesis that *A. nidulans*$_{\mathbf{BglC}}$ is a complex protein and it would need further cellular machinery to achieve proper folding. The sixth DE gene in *A. nidulans*$_{\mathbf{BglC}}$ was AN1296, found up-regulated at 2 and 8 h, and moderately up-regulated in *A. nidulans*$_{\mathbf{AbfA}}$. The orthologous of this gene in *S. cerevisiae* (TRL1) is required for tRNA splicing and for both splicing and translation of *hac1* mRNA in the UPR (Cherry et al., 2012). This result converges with our relative coverage data (**Figure S4**) that shows the unconventional splicing of *hacA*, suggesting the induction of UPR for heterologous protein production, as previously reported (Al-Sheikh et al., 2004; Guillemette et al., 2011; Heimel, 2014; Krishnan et al., 2014; Saloheimo et al., 2003; Zhou et al., 2016). On the other hand, *hacA* was down-regulated in *A. nidulans*$_{\mathbf{1542}}$ and *A. nidulans*$_{\mathbf{AbfA}}$ in the early induction period (**Additional file 2: Table S4**), demonstrating that the unconventional splicing of *hacA* is required for UPR activation, but does not necessarily lead to *hacA* overexpression (Heimel, 2014). Liu et al. (2014) also observed that mRNA levels of the *hacA* were not increased in *A. oryzae* recombinant strains, indicating that *hacA* splicing is not the only mechanism responsible for activating UPR in this organism (Liu et al., 2014).

A. nidulans$_{\mathbf{AbfA}}$ also presented two exclusives up-regulated genes, AN4583 and AN6145. Both genes are predicted as peptidyl-prolyl cis-trans isomerases (PPIases) (Cerqueira et al., 2014). These proteins accelerate folding of proteins, and these two PPIases may be interesting targets for genetic manipulation in order to improve protein secretion (Pemberton, 2006).

A. nidulans$_{\mathbf{1542}}$ showed only two exclusive DE genes. The first is a predicted 1,2-alpha-mannosidase (AN2045) with an ortholog (gh47-3) in *Neurospora crassa* reported to be an ER-associated ubiquitin-dependent protein with catabolic process and N-glycan processing (according to UniProt). The second was the AN8153 down-regulated at 2 and 8 h, which was not observed for the other strains which showed an up-regulation response at 2 h. This gene is predicted as ATP binding,

with nucleoside-triphosphatase activity and nucleotide binding activity (Cerqueira et al., 2014). In *S. cerevisiae* the best hit is CDC48, an ATPase; it is a subunit of a polyubiquitin-selective segregase complex involved in ERAD, with a role in mobilizing membrane-bound transcription factors by regulating ubiquitin/proteasome-dependent processing in macroautophagy (Cherry et al., 2012). Moreover, DE genes related to ERAD in this strain could partially explain the low levels of 1542 secretion. In *A. niger*, the knock-out of one ERAD factor improved the production of a heterologous beta-glucuronidase (Jacobs et al., 2009). DE genes related to ERAD indicated by the *A. nidulans* recombinant strains studied in this work seem to be potential targets for genetic manipulation in an attempt to improve heterologous protein secretion.

Tyo et al. (2012) identified biological mechanisms which alter the secretory pathway in response to the secretion of recombinant proteins of different sizes, *i.e*. a small protein (human insulin precursor) and a comparatively larger protein (α-amylase) in *S. cerevisiae* (Tyo et al., 2012). By perturbing the yeast secretory pathway with these proteins, they conclude that in order to maintain the optimal secretion ratio, protein folding rates must increase and oxidation rates must decrease (by repressing both ERAD and UPR). In this scenario, recombinant proteins would be folded slowly and without high cellular stresses. This would result in a longer/prolonged overall process which may be required due to the difficulty of protein folding (Tyo et al., 2012).

CONCLUSIONS

Despite the importance of *Aspergilli* as microbial cell factories with many industrial applications, only a limited number of genetic tools designed for overproduction and secretion of heterologous proteins are available. In our study, the secretion of heterologous proteins in *A. nidulans* was investigated. We performed a comparative RNA-seq based transcriptomic analysis of *A. nidulans* recombinant strains overproducing heterologous glycoside hydrolases.

The general response to heterologous protein production was a global transcriptional up-regulation response. At 2 h, we observed the unconventional splicing of *hacA*, which was normalized at 8 h. A transcriptomic profile for each strain was established based on the functional categorization of DE genes. From this analysis several proteins, including many chaperones, were DE and appeared to be interesting candidates for genetic manipulation, requiring a deeper understanding of their effects on heterologous protein secretion by *A. nidulans.*

Finally, by observing secretory pathway genes we found 17 DE genes distributed in the three recombinant strains. The most representative functional categories of these genes were used to build a secretory pathway profile of the transcriptional response. This study addressed the response to heterologous protein production in *A. nidulans* by associating transcriptomic and monitoring of three different recombinant proteins, which present different secretion levels. Thus, we may provide novel

genetic and cellular background and targets for genetic manipulation to comprehend and improve protein secretion by *A. nidulans.*

MATERIALS AND METHODS

Strains maintenance and cultivation conditions

A. nidulans$_{\mathbf{A773}}$ (pyrG89;wA3;pyroA4) was purchased from the Fungal Genetics Stock Center (FGSC, St Louis, MO). The strains *A. nidulans*$_{\mathbf{AbfA}}$, *A. nidulans*$_{\mathbf{BglC}}$ and *A. nidulans*$_{\mathbf{1542}}$ were previously transformed and tested by our research group, as described by Segato 2012 (Segato et al., 2012). 5-fluorotic acid (5-FOA) was purchased from Oakwood Products Inc (NC9639762), and all other chemicals were from Sigma–Aldrich, Megazyme and Fisher Scientific.

Vegetative cultures and conidia production were prepared by cultivation in minimal medium as described by Clutterbuck (Clutterbuck, 1992) and Pontecorvo (Pontecorvo et al., 1953). *A. nidulans* strains were cultivated in minimal medium containing 5% of 20× Clutterbuck salts, 0.1% of 1000× vitamins, 0.1% of 1000× trace elements, pH 6.5 and supplemented with pyridoxine (1 mg/L), uracil/uridine (2.5 mg/L each) whenever required. Minimal medium was supplemented with 1% of glucose for mycelium growth (growth medium) or with 2% maltose and 250 mM HEPES buffer (Sigma–Aldrich) for promoter activation and protein production (induction medium). Incubation was carried out at 37 °C.

Production and secretion of client proteins

Pre-cultures were prepared by inoculating fresh 10^8 spores/ml in 30 mL of minimal medium supplemented with 1% (w/v) glucose for the mycelium growth for 48 h at 150 rpm and 37 °C. Strains cultivation were performed in biological triplicates. The mycelium was washed with autoclaved distilled water, filtered with Miracloth, and then transferred to the induction medium. After defined periods for protein induction, the mycelium was collected, Miracloth filtered, dried, frozen in liquid nitrogen and stored at -80 °C. The medium was collected, centrifuged at 10,000 × g for 10 min prior to concentration by ultrafiltration (10 kDa cutoff Amicon), quantified by the Bradford method(Bradford, 1976), purity was assessed by SDS-PAGE (Shapiro and Maizel, 1969) and then used for biochemical studies.

Analytical assays

Enzymatic activity for beta-glucosidase GH3 (BglC) was performed with 4-nitrophenyl-β-D-glucopyranoside (Sigma–Aldrich) and for alpha-arabinofuranosidase GH51 (AbfA) with 4-

nitrophenyl-α-L-arabinofuranoside (Sigma–Aldrich). The enzymatic activity assays were performed by adding 40 µL of extracellular crude extract to 50 µL of each 5 mM substrate in 10 µL of 100 mM ammonium acetate buffer pH 5.5 followed by incubation for 45 min at 50 ºC. The enzymatic reactions were stopped with sodium carbonate. The absorbance was read at 405 nm with a Multimode Infinite M200 Reader (Tecan, SC), and activity was calculated using p-nitrophenol as standard.

Enzymatic activity of thermophilic mannanase GH5 (1542) was conducted with locust bean gum (Sigma–Aldrich) as substrate. The reaction was performed by adding 1 µg of proteins from concentrated extracellular crude extract to 50 µL of 1% (w/v) substrate in 50 mM ammonium acetate buffer pH 5.5 under incubation for 10 h at 87 ºC. The release of reducing sugars was measured with DNS acid (Miller, 1959), and absorbance was read at 540 nm with a Multimode Infinite M200 Reader (Tecan, SC) and compared to a glucose standard curve. Statistics calculation and plotting were carried out with GraphPad Prism v 5.0 Software (California, US).

The concentration of maltose was detected by high-performance liquid chromatography (HPLC) Agilent Infinity 1260 with IR detector 50C, Aminex column HPX-87P 300 mm x 7.8 mm at 50 °C and 0.5 mL/min of ultrapure Milli-Q water as eluent phase.

Intracellular protein analysis

Intracellular proteins were extracted from frozen mycelium after grounding in liquid nitrogen to a fine powder using a mortar and a pestle. The powder was gently suspended in 5 mL of extraction buffer (20 mM Tris-HCl pH 8.0; 0.05 % (w/v) Triton™ X-100 (Sigma-Aldrich), and 150 mM NaCl, containing protease inhibitors (2 mM PMSF and Protease Inhibitor Cocktail N221-1mL from Amresco), and centrifuged twice at 8000 rpm for 15 min at 4 ºC; then, the supernatant was collected for SDS-PAGE analysis (Shapiro and Maizel, 1969).

RNA extraction

Total RNA was extracted from frozen mycelium using the Quick-RNA™ MiniPrep kit (Zymo Research) according to the manufacturer's instructions. The integrity of extracted RNA was evaluated (RIN ≥ 8) on the Agilent Bioanalyzer 2100 and quantified using an ND-1000 NanoDrop (Thermo Scientific) spectrophotometer. Total purified RNA was stored at -80 °C until further processing.

RNA-seq data analysis

For RNA-seq sample preparation, the total RNA from the four *A. nidulans* strains (three recombinant strains and the parental strain) cultivated for 2 and 8 h in biological triplicates, resulted

in twenty-four samples for library preparation, using TruSeq Stranded mRNA Library Prep Kit v2 (Illumina) according to the manufacturer's instructions. Sequencing libraries were prepared and sequenced using Illumina HiSeq 2500 at CTBE NGS sequencing facility. Approximately 148 million 100 bp paired-end reads were obtained for *A. nidulans*$_{\mathbf{A773}}$, *A. nidulans*$_{\mathbf{AbfA}}$, *A. nidulans*$_{\mathbf{BglC}}$ strains and, 130 million reads were obtained for *A. nidulans*$_{\mathbf{1542}}$ strain, representing 115 GB. Reads were quality checked and filtered using FASTQC and Trimmomatic (Bolger et al., 2014), respectively, and rRNA contamination was assessed and removed using sortmeRNA (Kopylova et al., 2012). The rate of rRNA contamination was lower than 16% in all samples, except in one replicate of AbfA 8h (**Additional file 3: Table S5**). QC reads were aligned to *Aspergillus nidulans* genome available at AspGD (http://www.aspgd.org/) using Tophat2 (Kim et al., 2013), and concordant pair alignment rate varied between 84 and 93%. An acceptable replicate agreement was obtained for all conditions, as showed by the PCA analysis and clustering of the samples data.

For differential expression analysis, pairwise comparisons between each strain and the control strain *A. nidulans*$_{\mathbf{A773}}$ were performed using DESeq2 R/Bioconductor package (Love et al., 2014), applying a log2 fold change ≥ 2 or ≤ -2 and an adjusted p-value ≤ 0.01 as thresholds. For the secretion pathway analysis, it was used a log2 fold change ≥ 1 or ≤ -1 and an adjusted p-value ≤ 0.01 as thresholds. Graph constructions were performed using GraphPad Prism v 5.0 Software (California, US) and the online tool for Venn diagrams - Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).

Functional annotation

For functional annotation of DE genes identified by RNA-seq analysis, lists of genes were generated by filtering the results with log 2 FC $\leq$ -1.0 or $\geq$ 1.0 and adjusted p-value ≤ 0.01. The gene expression values of the recombinant strains were compared to the control strain *A. nidulans*$_{\mathbf{A773}}$. The gene lists obtained were then used for functional annotation using FunCat (Functional Catalogue) (Ruepp, 2004).

The selected genes for annotation with FunCat were faced with the database of the reference genome of *A. nidulans* and applied statistical Fisher test with p-value ≤ 0.05 as threshold. The enriched functional categories and subcategories were used for comparisons and graphics generation. Data analysis was performed using GraphPad Prism v 5.0 Software (California, US).

Secretion pathway analysis

A list of 374 secretory pathway genes was generated based on Liu et al. (2014), which defined a list with the functional protein secretory components from *A. oryzae* using the secretory model of

S. cerevisiae as scaffold (Liu et al., 2014). This list was further adapted to filamentous fungi by adding *A. oryzae* orthologous of the secretory components reported for other *Aspergillus* species, such as *A. nidulans* and *A. niger*. The homologous genes in *A. nidulans* reported in AspGD resources were then used to generate a list with 374 genes.

DE genes of the secretory pathway were functionally annotated with FunCat, and the most representative functional categories were used to build a secretory pathway profile of three recombinant strains. To generate this profile, the FC values of the DE genes involved with secretion pathway were used to create heatmaps using R.

Real-Time PCR (qPCR) analysis

To quantify the expression of selected genes, quantitative real-time PCR (qRT-PCR) was performed. For this analysis, 2 µg of pure RNA was used for cDNA synthesis using the First Strand cDNA kit Maxima™ Synthesis (Thermo Scientific), according to the manufacturer's instructions. The cDNA was 1/50 diluted and used for analysis in the ViiA™ 7 Real-Time PCR System, using Maxima SYBR™ Green (Thermo Scientific) for signal detection, in accordance with the manufacturer's instructions. The gene encoding β-tubulin (AN6838) was used as endogenous control for being a well stable gene in filamentous fungi (Llanos et al., 2015).

The PCRs conditions were: 95 °C for 30 s; followed by 40 cycles at 95 °C for 10 s and 60 °C for 60 s. The melting curve was analyzed with ViiA™ 7 Software (Thermo Scientific) to confirm the presence of only one amplicon, according to the Tm expected for each gene. Gene expression values were calculated according to the 2-ΔΔCT method (Livak and Schmittgen, 2001). Primers used in the qPCR experiments are described in Additional file 4 (**Additional file 4: Table S6**). Data analysis was performed using GraphPad Prism v 5.0 Software (California, US).

Data availability

The datasets generated during and/or analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository, GSE101522, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE101522.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

ARLD conceived and designed the experiments. FC, MVR, MPZ participated in design of the study and performed the RNAseq experiments. GFP filtered the RNAseq raw data and generated the differential expressed genes lists. FC, MVR, MPZ, FJC, GFP and ARLD analyzed the RNAseq data. FC, FMS and FJC helped to interpret the experimental data. FC and ARLD drafted the manuscript. ARLD, CRFT, FMS and GFP revised the manuscript. All authors read and approved the final manuscript.

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FIGURE CAPTIONS

Figure 1. Physiological parameters and heterologous protein production by *A. nidulans* recombinant strains. A) *A. nidulans*$_{AbfA}$; B) *A. nidulans*$_{BglC}$; C) *A. nidulans*$_{1542}$. Physiological parameters (top): mycelium dry weight (g), final pH of the cultivation medium, extracellular proteins (mg/mL). Enzymatic activity of the *A. nidulans* strains in different cultivation periods (center). The highest enzymatic activity in each cultivation was considered 100%. SDS-PAGE of the heterologous proteins secreted by the recombinant *A. nidulans* strains in different cultivation periods (bottom). On each lane, 10 µg of proteins were applied. The target proteins are indicated by an asterisk. The red box indicates a native intracellular beta-glucosidase detected after 48 h of cultivation. The band corresponding to the 1542 protein was not detected in the SDS-PAGE (bottom at right). Error bars indicate the standard deviation of three replicates.

Figure 2. Gene expression profile of *A. nidulans* recombinant strains. Expression levels were determined by qPCR in different cultivation periods and expressed on a logarithmic scale as folding changed. A) Gene expression profile of the heterologous genes *abfA*, *bglC* and *1542*. B) Expression levels of *bipA* in *A. nidulans*$_{AbfA}$, *A. nidulans*$_{BglC}$ and *A. nidulans*$_{1542}$. C) Expression levels of sec63 in *A. nidulans*$_{AbfA}$, *A. nidulans*$_{BglC}$ and *A. nidulans*$_{1542}$. *tubC* was used as the reference gene and the strain *A. nidulans*$_{A773}$ as the experimental control. The ΔΔCt method was used for gene expression calculation. Asterisks indicate statistically different results (two-way ANOVA and Bonferroni post-test p-value<0.05). The strains were cultivated as described in the Materials and Methods section. Results are expressed as mean values of three biological replicates and the error bars indicate standard deviation.

Figure 3. Global analysis of differentially expressed genes. A) Bar chart representing differentially expressed (DE) genes by the *A. nidulans* recombinant strains. Rectangles above the bars present the number of up and downregulated genes. RNA-seq data of each recombinant strain were compared to the control strain *A. nidulans*$_{A773}$ and (log 2) fold change ≤ -2.0 or ≥ 2.0, and adjusted p-values ≤ 0.01 were used as thresholds. B) Venn diagram representing DE genes analyzed by the RNA-seq at 2 and 8 h of cultivation for the three *A. nidulans* recombinant strains. The Venn diagram was created using the online tool Venny 2.1.

Figure 4. Functional annotation of the transcriptomic data of *A. nidulans* recombinant strains. MIPS FunCat term annotations of *A. nidulans*$_{\mathbf{1542}}$, *A. nidulans*$_{\mathbf{AbfA}}$ and *A. nidulans*$_{\mathbf{BglC}}$ show a broad variety of biological processes associated with DE genes of the *A. nidulans* recombinant strains.

Analysis of the functional categories from global transcriptomic data was performed with Funcat (The Functional Catalogue). Functional categories of the heatmap represent the number of DE genes found in the transcriptomic data based on the reference genome annotation. The heatmap was created with the online software from the Broad Institute – Morpheus.

Figure 5. **Differentially expressed genes common in all recombinant strains**. (A) Hierarchical clustering fell into three major clusters. (B) A schematic representation of each pattern of transcriptional change: up (increased at 2 and 8 h), down to up (decreased at 2 h and increased at 8 h), and up to down (increased at 2 h and decreased at 8 h). Circles: 10 genes; squares: 8 genes; triangles: 12 genes. Gray symbols: 2 h. Black symbols: 8 h.

Figure 6. Analysis of genes related to the secretory pathway. A) Heatmap and representing the 17 DE genes related to the secretion pathway in A. nidulans. B) Venn Diagram representing the 17 DE genes related to the secretion pathway. No gene was found in common to all the recombinant strains. Venn diagram was created using the online tool Venny 2.1. C) Functional annotations of differentially expressed genes related to the secretory pathway of recombinant A. nidulansAbfA, A. nidulansBglC and A. nidulans1542. The analysis of functional categories was performed with FunCat (The Functional Catalogue). The functional categories graph values represent the number of altered genes found (abs set) in the transcriptomic data based on the values of the reference genome annotation. All functional subcategories with at least five differentially expressed genes are represented. A. nidulans strains were cultivated as described in the Materials and Methods section. Heatmaps were created with online software from Broad Institute – Morpheus.

Figure 1

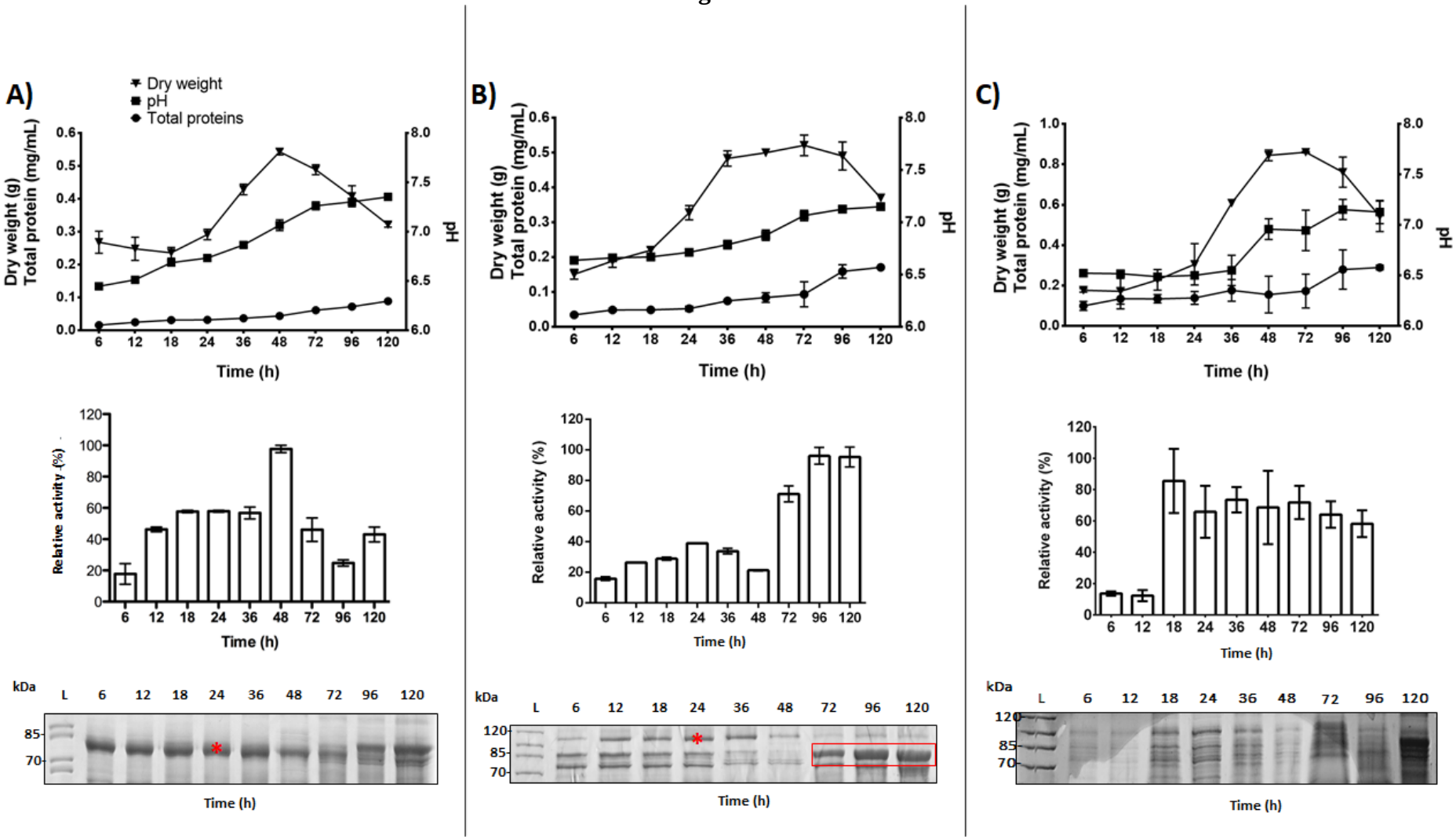

A)
Dry weight
pH
Total proteins
Dry weight (g)
Total protein (mg/mL)
pH
Time (h)
Relative activity (%)
Time (h)
kDa
L 6 12 18 24 36 48 72 96 120
85
70
Time (h)
B)
Dry weight (g)
Total protein (mg/mL)
pH
Time (h)
Relative activity (%)
Time (h)
kDa
L 6 12 18 24 36 48 72 96 120
120
85
70
Time (h)
C)
Dry weight (g)
Total protein (mg/mL)
pH
Time (h)
Relative activity (%)
Time (h)
kDa
L 6 12 18 24 36 48 72 96 120
120
85
70
Time (h)

Figure 2

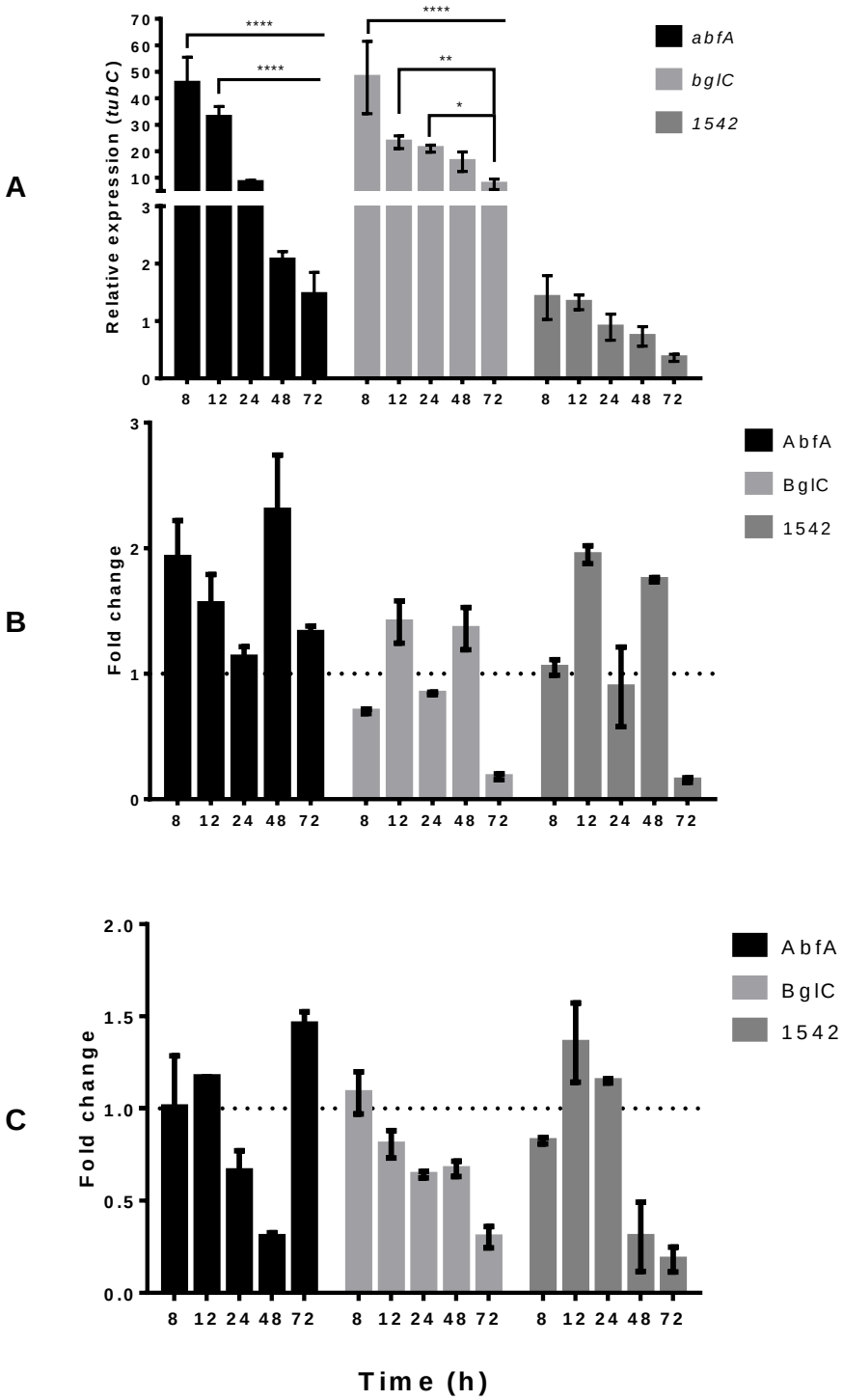
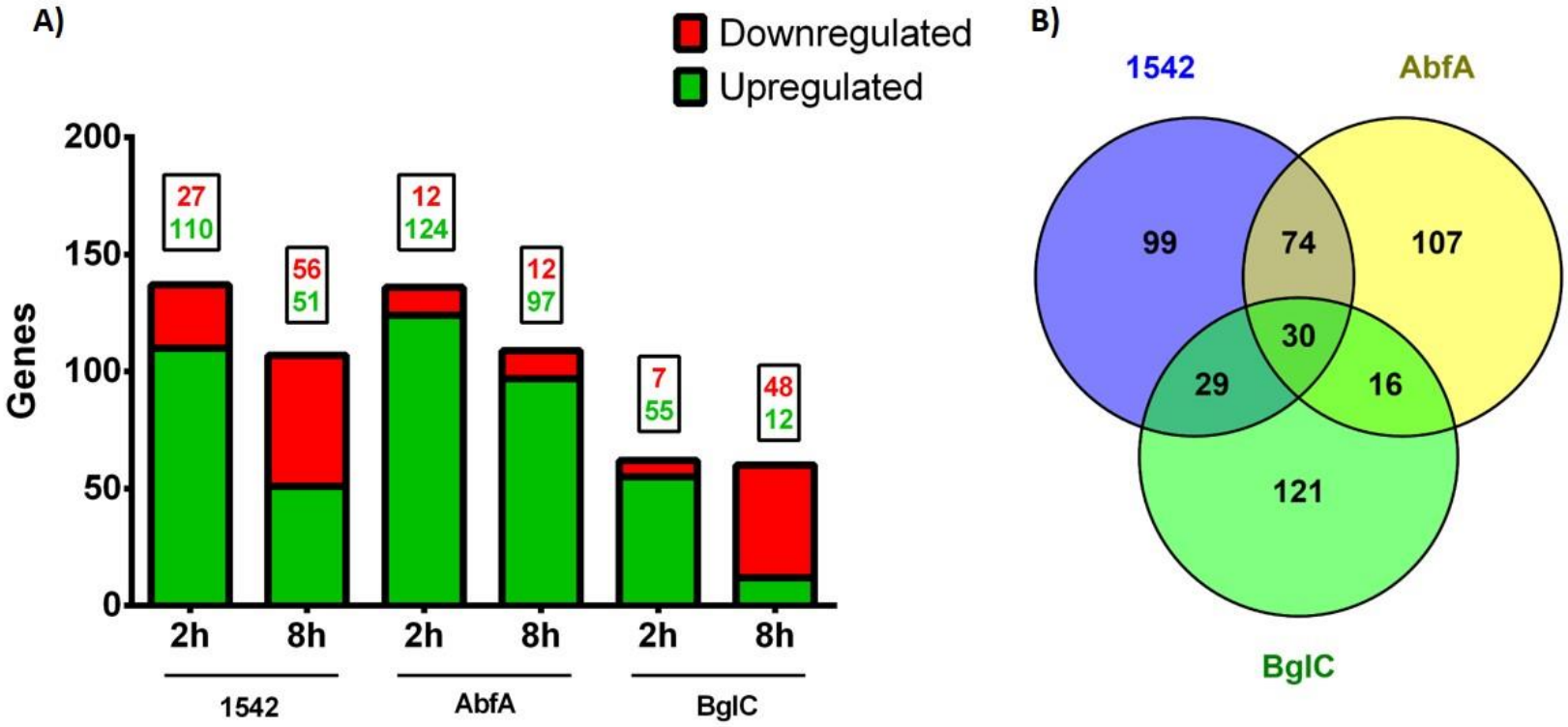

A
Relative expression (tubC)
abfA
bglC
1542
B
Fold change
AbfA
BglC
1542
C
Fold change
AbfA
BglC
1542
Time (h)

Figure 3


A)
Downregulated
Upregulated
Genes
200
150
100
50
0
27
110
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51
12
124
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48
12
2h
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B)
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AbfA
BglC
99
74
107
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29
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121

Figure 4

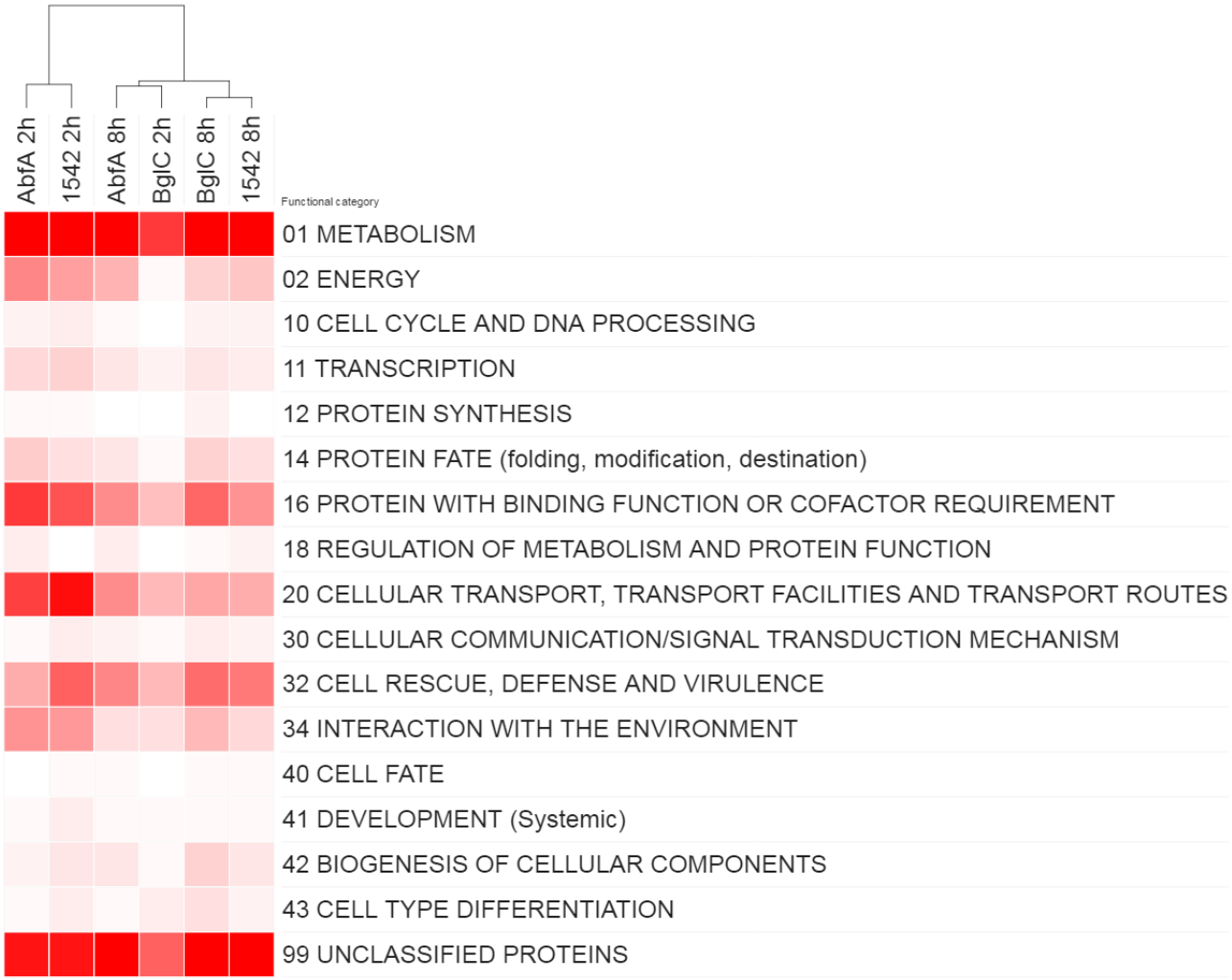

AbfA 2h
1542 2h
AbfA 8h
BglC 2h
BglC 8h
1542 8h
Functional category
01 METABOLISM
02 ENERGY
10 CELL CYCLE AND DNA PROCESSING
11 TRANSCRIPTION
12 PROTEIN SYNTHESIS
14 PROTEIN FATE (folding, modification, destination)
16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT
18 REGULATION OF METABOLISM AND PROTEIN FUNCTION
20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES
30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM
32 CELL RESCUE, DEFENSE AND VIRULENCE
34 INTERACTION WITH THE ENVIRONMENT
40 CELL FATE
41 DEVELOPMENT (Systemic)
42 BIOGENESIS OF CELLULAR COMPONENTS
43 CELL TYPE DIFFERENTIATION
99 UNCLASSIFIED PROTEINS

Figure 5

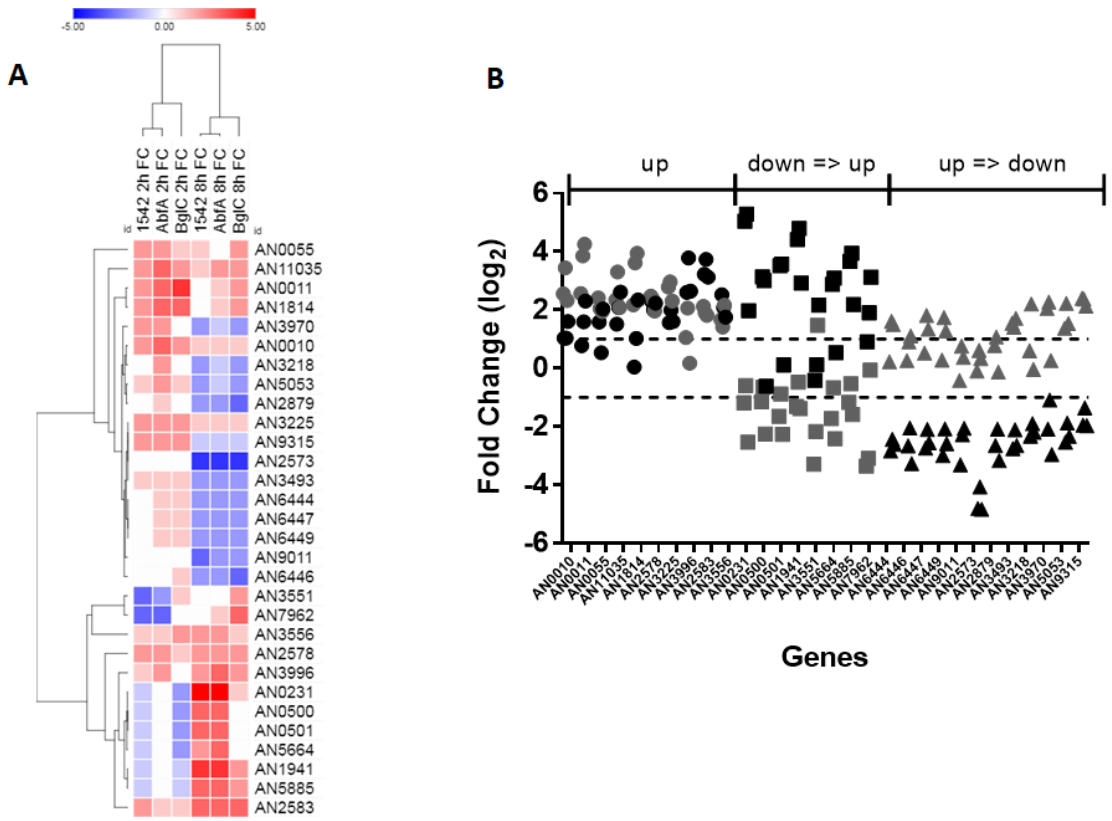
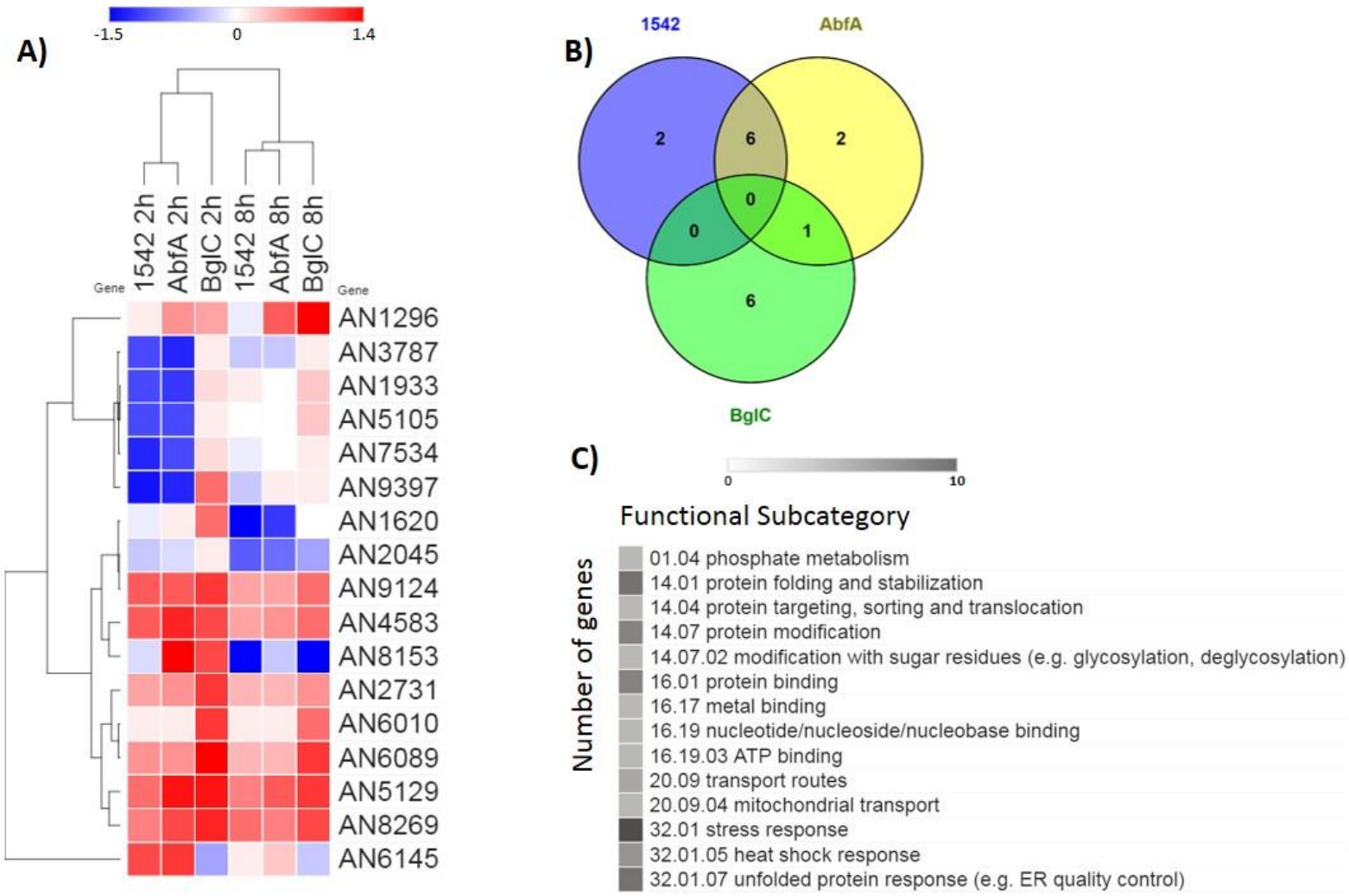

A
-5.00
0.00
5.00
1542 2h FC
AbfA 2h FC
BglC 2h FC
1542 8h FC
AbfA 8h FC
BglC 8h FC
id
AN0055
AN11035
AN0011
AN1814
AN3970
AN0010
AN3218
AN5053
AN2879
AN3225
AN9315
AN2573
AN3493
AN6444
AN6447
AN6449
AN9011
AN6446
AN3551
AN7962
AN3556
AN2578
AN3996
AN0231
AN0500
AN0501
AN5664
AN1941
AN5885
AN2583
B
up
down => up
up => down
Fold Change (log2)
6
4
2
0
-2
-4
-6
Genes

Figure 6


A)
-1.5
0
1.4
1542 2h
AbfA 2h
BglC 2h
1542 8h
AbfA 8h
BglC 8h
Gene
Gene
AN1296
AN3787
AN1933
AN5105
AN7534
AN9397
AN1620
AN2045
AN9124
AN4583
AN8153
AN2731
AN6010
AN6089
AN5129
AN8269
AN6145
B)
1542
AbfA
BglC
2
6
2
0
0
1
6
C)
0
10
Functional Subcategory
Number of genes
01.04 phosphate metabolism
14.01 protein folding and stabilization
14.04 protein targeting, sorting and translocation
14.07 protein modification
14.07.02 modification with sugar residues (e.g. glycosylation, deglycosylation)
16.01 protein binding
16.17 metal binding
16.19 nucleotide/nucleoside/nucleobase binding
16.19.03 ATP binding
20.09 transport routes
20.09.04 mitochondrial transport
32.01 stress response
32.01.05 heat shock response
32.01.07 unfolded protein response (e.g. ER quality control)