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Summary

Penicillium glabrum is a filamentous fungus frequently involved in food contamination. Numerous environmental factors (temperature, humidity, atmosphere composition, etc.) or food characteristics (water activity, pH, preservatives, etc.) could represent potential sources of stress for micro-organisms. These factors can directly affect physiology of these spoilage micro-organisms: growth, conidiation, synthesis of secondary metabolites, etc. This study investigates the transcriptional response to temperature in *P. glabrum*, since this factor is one of the most important for fungal growth. Gene expression was first analysed by using suppression subtractive hybridization to generate two libraries containing 445 different up- and down-regulated Expressed Sequenced Tags (ESTs). Expression of these ESTs was then assessed for different thermal stress conditions, with cDNA microarrays, resulting in the identification of 35 and 49 significantly up- and down-regulated ESTs, respectively. These ESTs encode heat shock proteins, ribosomal proteins, superoxide dismutase, trehalose-6-phosphate synthase and a large variety of identified or unknown proteins. Some of them may be potential molecular markers for thermal stress response in *P. glabrum*. To our knowledge, this work represents the first study of the transcriptional response of a food spoilage filamentous fungus under thermal stress conditions.

Introduction

Fungi are ubiquitous micro-organisms often associated with spoilage and biodeterioration of a large variety of foods and feedstuffs. *Penicillium* is one of the most widespread fungal genera isolated from food products. In addition to the economic losses caused by these contaminants, many *Penicillium* species can produce mycotoxins that represent a potential health risk for humans and animals (Pitt & Hocking, 1997; Samson *et al.*, 2004). Due to its ability to disperse a large number of spores in the environment, *P. glabrum* is very frequently encountered in the food manufacturing industry (Pitt & Hocking, 1997). This fungus is responsible for spoilage of many different products, such as cheese (Northolt *et al.*, 1980), stored cereals (Borjesson *et al.*, 1992), maize (Mislivec & Tuite, 1970), rice (Kurata *et al.*, 1968), jam (Udagawa *et al.*, 1977), commercially marketed chestnuts (Overy *et al.*, 2003), soda (Ancasi *et al.*, 2006) and mineral water (Cabral & Fernandez Pinto, 2002). Numerous intrinsic factors (water activity, pH, preservatives, etc.) in foods, provide ideal conditions for micro-organisms to develop. Combined with extrinsic factors (temperature, humidity, atmosphere composition, etc.), modification of these conditions are potential sources of stress that can directly affect the physiology (growth, sporulation, synthesis of secondary metabolites, etc.) of spoilage micro-organisms. *In vitro* data obtained with different *Penicillium* strains, have shown significant variation in the production of secondary metabolites among substrate (Chang *et al.*, 1991; Kokkonen *et al.*, 2005). Environmental conditions may also modify mycotoxin production as reported in *Fusarium* species (Magan *et al.*, 2002). Some modification, such as osmotic stress have been shown to influence the synthesis of secondary metabolites in *P. notatum* (Fiedurek, 1997).

Temperature and water activity are the most important factors that determine the ability of moulds to grow (Dantigny *et al.*, 2005). Fungi and more generally, every organism in each kingdom, have developed strategies to respond rapidly to environmental fluctuations. Among them, elevated temperatures are one of the foremost and best-known stress inducers at the cellular level. The general response to thermal stress is characterised by acquired thermotolerance, which increases survival capacity during subsequent exposure to high temperatures (Piper, 1993). Response to thermal stress has been described as a tolerance mechanism against adverse growth conditions and associated with synthesis of heat shock proteins (HSPs) (Lindquist, 1986). This mechanism aims to stabilize cell metabolism and prevents the accumulation of denatured proteins which can be toxic for the cell. Heat shock response appears to be widespread, spanning many taxonomic ranks and is found in drosophila (Ashburner & Bonner, 1979), rabbit (Cosgrove & Brown, 1983), *E. coli* (Yamamori & Yura, 1982), *Saccharomyces cerevisae* (McAlister & Finkelstein, 1980), *Aspergillus nidulans* (Newbury & Peberdy, 1996), etc. To our knowledge, no data are available on the heat shock transcriptional response of food-contaminating moulds, such as *P. glabrum*. From a food industry perspective, understanding the effects of various factors on the growth and physiology of a spoilage mould, is crucial to better manage the risk of alteration and toxicity of food products. It would therefore be useful to gain more insight on the mechanisms involved in thermal stress and to identify the genes that are differentially regulated in response to thermal stress.

In the present study, the effects of temperature on the transcriptional response of *P. glabrum* was investigated by using two complementary techniques: suppression subtractive hybridization (SSH) and cDNA microarrays. Transcriptional response to heat shock was compared between thermal stress conditions (40°C, 120 min) and the optimal growth temperature of 25°C. SSH is a powerful method based on suppression PCR where subtracted

cDNA libraries can be constructed to identify differentially expressed genes in response to an experimental variation in physiological, environmental or other factores (Diatchenko *et al.*, 1996). This method has been previously used in fungi to isolate differentially expressed genes associated with different morphologies or different growth conditions. It has been applied in *Aspergillus nidulans* to characterise genes specifically expressed in conidiating cultures and mature conidia (Osherov *et al.*, 2002). SSH has been also used to investigate the differential gene expression between mycelium and yeast forms of the human pathogens *Penicillium marneffei* (Liu *et al.*, 2007) and *Paracoccidioides brasiliensis* (Marques *et al.*, 2004) with respect to temperature (25° or 37°C).

Expressed Sequenced Tags (ESTs) isolated by SSH, were then investigated, in different thermal stress conditions, by assessing their expression with the cDNA microarray technique and confirmed with real-time reverse transcription PCR (qRT-PCR) on few selected ESTs. The advent of cDNA microarrays has provided the possibility of analysing the global changes in gene expression that occur in a cell, under different conditions (Shalon *et al.*, 1996). Transcriptional studies using whole-genome microarrays have been conducted on a wide range of environmental conditions (e.g. thermal, osmotic and oxidative stresses) in the yeasts *S. cerevisiae* (Gasch *et al.*, 2000; Causton *et al.*, 2001; Sakaki *et al.*, 2003) and *Schizosaccharomyces pombe* (Chen *et al.*, 2003). As the whole genome of *P. glabrum* is not available, we constructed a custom-made cDNA microarray and we report here the results obtained. This work represents, to our knowledge, the first study investigating the transcriptional response of a food spoilage fungus under thermal stress.

Methods

Biological material and culture conditions

The fungal strain used in this study was isolated from contaminated aromatized mineral water and precisely characterised as *Penicillium glabrum* (Wehmer) Westling according to the reference method used for classifying *Penicillium* species (Pitt, 2000). It was registered as LMSA 1.01.421 in the fungi collection ''Souchothèque de Bretagne'' (Brest, France). For conservation and collecting spores, the fungus was cultured in tubes of potato dextrose agar medium (PDA) (Difco Laboratories) at 25°C. Spores were collected from seven-day-old mycelium by adding, 2 mL of sterile water containing 0,01 % of Tween 80 (Sigma-Aldrich) to each tube and flooding with agitation at 250 rpm. Mycelia were cultured from a suspension of $5 \cdot 10^6$ spores of *P. glabrum* inoculated in 250 ml Erlenmeyer flasks containing 50 ml of potato dextrose browth medium (PDB) (Difco Laboratories). For suppression subtractive hybridization, *P. glabrum* was exposed briefly to two different conditions: stress (40°C, 120 min) and control (25 °C), hereafter referred as S and C, respectively. In both case, mycelia were first grown in 50 ml of PDB medium shaken at 25°C, 120 rpm for 48 h, then sterile-filtered, transferred into 50 ml of sterile PDB media and grown for 120 min at 25°C or 40°C shaking at 120 rpm. Mycelia were then aseptically harvested by filtration, washed thoroughly with sterile water, quickly frozen in liquid nitrogen and stored at −80°C. For microarray experiments, five different thermal stress conditions were studied, as defined in a reduced central composite design (CCD) (Box *et al.*, 1978): S1 (33 °C, 42 min), S2 (33 °C, 198 min), S3 (40 °C, 120 min), S4 (47°C, 42 min) and S5 (47°C, 198 min) (Fig. 1). These five culture conditions were defined from a previous CCD used for physiological investigations of thermal stress in *P.glabrum* (unpublished data). For each condition, mycelia were grown 48 h

in triplicate (a, b and c), heat shocked according to the different conditions and harvested following the method previously described above. Mycelia referred as ''Reference''(R) and ''Control'' (C) were also grown in triplicate, at 25°C, in order to constitute respectively the unique reference pool (pool R) or the Ca, Cb, Cc samples used for statistical comparison with results obtained under stress conditions.

Suppressive Subtractive Hybridization

For each sample, the frozen mycelium was reduced to a fine powder by grinding in a mortar containing liquid nitrogen and total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was quantified using Nanodrop spectrophotometer (Labtech) and their integrity was verified with Bioanalyser (Agilent Technologies) and RNA Nano LabChip (Agilent Technologies). Poly $(A)^+$ RNAs were then isolated with PolyATtract kit (Promega) according to the manufacturer's instructions. The Poly $(A)^+$ RNAs were then precipitated overnight at $-20°C$ with 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol. Poly $(A)^+$ RNAs were then washed twice in 75 % (v/v) ethanol and finally re-suspended with 12μL of RNase-free water. The Poly $(A)^+$ RNAs extracted were then quantified using Nanodrop spectrophotometer (Labtech).

Two reciprocal subtractive cDNA libraries were constructed by SSH on poly $(A)^+$ RNAs corresponding to C (25°C) and S (40°C, 120 min) conditions using the PCR-Select cDNA subtraction kit (BD Biosciences–Clontech) according to the manufacturer's instructions. After subtraction, isolated cDNA fragments were amplified by PCR with Advantage cDNA PCR kit

(BD Biosciences–Clontech), purified with QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T easy vector (Promega) in JM 109 Bacteria (Promega) according to the manufacturer's instructions. Bacterial clones of both forward and reverse libraries were cultured for 24 h at 37°C, in Luria-Bertani (LB) medium (supplemented with 100 mg ampicillin l^{-1}). Plasmids were then extracted from these bacterial clones using an alkaline lysis plasmid minipreparation (Birnboim & Doly, 1979). Positive clones containing a single insert were then selected after *Eco*RI digestion and 1% agarose gel electrophoresis.

DNA manipulations and sequence analysis

The cDNAs isolated from positive clones, were sequenced with BigDye Terminator chemistry on an AB 3130 xl sequencer (Applied Biosystems). All sequencing was performed with the Sp6 primer (5'-GATTTAGGTGACACTATAG-3', Tm = 56°C) at a concentration of 5 µM for each reaction. The obtained raw sequences were trimmed using Phred software (Ewing *et al.*, 1998), plasmid sequences were eliminated with SeqClean software (TIGR software tools, http://www.tigr.org/tdb/tgi/software). The cleaned sequences were then compared using BLASTx program (Altschul *et al.*, 1990) with non-redundant GenBank and Swiss-Prot protein sequence databases. Sequences of cDNA contained in both subtracted libraries were annotated and the corresponding ESTs were classified based on the function of their putative encoded proteins. Functional classification used in this work comes from a similar study on *Paracoccidioides brasiliensis* (Felipe *et al.*, 2005) and is originally based on MIPS clustering (Ruepp *et al.*, 2004).

cDNA microarray construction

In order to normalise the amount of cDNA used for microarray, the bacterial clones used for the subtracted libraries, were cultured in LB medium (with 100 mg ampicillin l^{-1}) for 6 h at 37°C, at 140 rpm. Twenty microliters of each culture were diluted in 80 µL of sterile water, then incubated at 95°C for 8 min and 4°C for 10 min. Two microliters of each lysed bacterial clone solution, were used in duplicate as cDNA template for PCR amplification in 50 µL of final reaction volume with an initial denaturation at 94 °C, 3 min , 40 cycles of 94°C for 20 s , 56°C for 30 s, 68°C for 2 min and a final elongation step at 68°C for 5 min. The reaction was performed with Taq DNA polymerase Kit (Amersham-Pharmacia Biotech): 3 U Taq polymerase per reaction, 0.25 mM dNTP, 0.01 µM T7 primer (5'-TAATACGACTCACTATAGGG-3', Tm = 56°C) and 0.01 µM Sp6 primer (5'-GATTTAGGTGACACTATAG-3', Tm = 56°C). PCR product duplicates were then pooled. Amplified cDNAs were purified using Multiscreen plate MSNU 03010 (Millipore) and re-suspended in 3X saline sodium citrate (SSC) (Invitrogen) containing 1.5 M betaine (Sigma-Aldrich). A semi-quantitative estimation was then performed on agarose gel electrophoresis with Smart Ladder SF (Eurogentec) in order to validate for each clone, the presence of single amplified cDNA band and its normalised intensity in each clone. Purified PCR products, were then spotted in triplicate onto GAPS II coated slides (Corning) with Eurogrid Spotter (Eurogentec).

Target preparation and hybridization

For target preparation, total RNAs were extracted from biological triplicates (a, b, c) of each mycelium sample grown in control (C and R) and stress conditions (S1, S2, S3, S4, S5),

as previously described. Total RNA from each sample was extracted with Trizol (Invitrogen) and genomic DNA leftovers were eliminated by DNase I treatment with RNase-free DNase set (Qiagen) and RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's instructions. Purified total RNAs were then analysed with Nanodrop spectrophotometer (Labtech) and Bioanalyser (Agilent Technologies). Total RNAs extracted from the 3 distinct samples Ra, Rb, Rc were then pooled in order to form a single reference pool (pool R). This pooled sample was used as a unique reference for hybridizations against samples corresponding to different conditions (C, S1, S2, S3, S4, S5). Five hundred nanograms of each RNA sample were used to prepare cDNAs by Reverse Transcription (RT). Those cDNAs were then 'in vitro transcribed'' and labelled in amplified RNA (aRNA) targets with Amino-Allyl MessageAmp II kit (Ambion) and Cy3 / Cy5 dye (Amersham-Pharmacia Biotech), according to the manufacturer's instructions.

Before target hybridization, the cDNA microarray slides were first hydrated (10 s), dried (10 s) and fixed for 20 s at 60 000 μJoules with Spectrolinker UV Crosslinker (Spectroline). Microarrays were then agitated 15 min at 100 rpm in a solution containing 130 mL 1-methyl-2-pyrrolidone (Sigma-Aldrich), 0.22 M succinic anhydride (Sigma-Aldrich) and 10 mL 1 M borate buffer, pH 8. The arrayed cDNA fragments were then denatured by placing the slides in milliQ water at 95°C for 2 min, washed in 95 % (v/v) ethanol (Prolabo) and immediately dried by centrifugation at 700 rpm for 3 min at room temperature. Slides were then pre-hybridized by incubating at 42°C for 1 h in a solution containing 3X SSC, 0.3 % SDS, 1 % Bovine Serum Albumine (BSA) sterilized by 0.22 μm filtration. Microarrays were then washed in 5 different milliQ water baths and then immediately dried by centrifugation at 700 rpm for 3 min at room temperature.

Competitive hybridizations were then realised by first mixing 500 ng of Cy3-labeled aRNA targets (S) and 500 ng of Cy5-labeled aRNA targets (C), denaturing 2 min at 100°C and incubating 30 min at 37°C. Mixed targets were then transferred onto the microarray surface, a cover slip was applied on top and microarrays were then incubated for 12 h at 42°C. After hybridization, microarrays were rinsed for 2 min at 100 rpm in four successive baths (respectively 2 X SSC, 0.1 % SDS; 1 X SSC, 0.2 X SSC twice) and immediately dried by centrifugation at 700 rpm for 3 min at 25°C.

Microarray data analysis

Hybridized slides were scanned at 10-µm resolution with ScanArray 4000 (Perkin Elmer) and ScanArray Express software (Perkin-Elmer). Pixel fluorescence intensity and differential expression ratios were determined from the picture files (.tiff) for both channel (Cy3 and Cy5) by using GenePix Pro 6.0 software (Molecular Devices Corporation). Lowess fitness regression (Yang *et al.*, 2002) was applied for global normalisation of raw expression ratios. Normalised data for the 913 cDNAs were $\log_2$ transformed and where an EST was represented by more than one cDNA, the median value was recorded. A statistical analysis of the results obtained for each experimental condition, was carried out with Microsoft Excel. A global ANOVA on results obtained for C, S1 and S2 conditions followed by a LSD test, were applied in order to isolate ESTs which were, significantly ($p \leq 0.05$) differentially expressed between S1-C and S2-C. Among these ESTs, only those with S:C expression ratios, corresponding with differences of at least 0.7 $\log_2$ (i.e. 1.62 fold change) were selected to make the up- and down-regulated EST lists.

qRT-PCR experiments

Reverse transcription (RT) was performed on 50 µg of poly (A)$^+$ RNA purified from total RNA samples used for preparing microarray labeled targets. ImProm-II Reverse Transcription System (Promega) was used according to the manufacturer's instructions, Real-time PCR (qPCR) experiments were performed with a Mini-Opticon thermocycler (Bio-Rad). Experiments were performed on 1 µl of two twice-diluted cDNA samples with 0.5 M each primer and 7.5 µl 2X iQ SYBR Green Supermix (Bio-Rad) in a final reaction volume of 15 µl. Cycling conditions were 2 min at 50°C, 2 min at 95 °C, 40 cycles (15 s at 95 °C, 1 min at 60 °C) and 15 s at 95 °C.

In the first step of the qRT-PCR experiments, the internal normalisation control was determined. A set of eight genes was chosen and composed of β-actin (frequently used as a housekeeping gene) and seven other genes presenting relative thermal stability according to microarray results. This group contained seven heterogeneous genes encoding adenosine kinase, 14-3-3 protein homolog, FAD-dependent sulfhydryl oxidase, copper-fist DNA binding domain protein, ATP synthase gamma chain, pre-mRNA splicing factor ini1 and S-phase kinase-associated protein 1A. The sequences of these genes inr *P. glabrum* were unavailable in public databases. Nevertheless, for β-actin gene, partial sequences were obtained from a previous study. Moreover, the seven other genes were previously cloned and partially sequenced for SSH experiments.

Another set of ten ESTs was also selected from the microarray analysis in order to assess their expression by qRT-PCR and validate our results. These ESTs were selected because their expression appeared to vary significantly with thermal stress. These ESTs encode for

heat shock protein 70, heat shock protein 98, trehalose-6-phosphate synthase, import translocase tim 50, NADP alcohol dehydrogenase, DMP phosphatase, hypothetical protein AN5480.2, hypothetical protein EAL 90164.1, AAA family ATPase, transcriptional activator HAA1.

For each gene, a pair of specific primers was designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) (Rozen & Skaletsky, 2000) in order to produce 120-180 bp amplicons (Table 1a and 1b).

Each primer pair was first tested with qRT-PCR in order to check that its PCR amplification efficiency was superior than 0.90. For each gene, the melting curve profile was first inspected in order to verify amplification specificity. Standard curves were then determined by plotting the threshold cycle (Ct) values of serial 10-fold dilutions (5, 50, 500, 5000) of a calibrator cDNA template as a linear function of the log of the dilution factor. The calibrator cDNA was obtained by reverse transcription on a mix of different stressed or control RNAs. Expression values were then calculated with the threshold cycle (Ct) of the fluorescence measures obtained. Normalised data were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) using the expression of an internal control gene. Microarray and qRT-PCR results obtained for the ten selected genes, were then compared using a Bland–Altman plot (Bland & Altman, 1996) as implimented by others (Steenman *et al.*, 2005).

Results

Subtracted cDNA libraries

More than 1300 clones were randomly selected from the forward and reverse cDNA subtracted libraries constructed for *Penicillium glabrum* after comparison between C (25 °C) and S (40 °C, 120 min). Among them, 1056 positive clones were sequenced and finally 913 clean and useful cDNA sequences were obtained. Using BLASTx on GenBank and Swiss-Prot protein databases, 80 % of these sequences matched with ORFs of other fungi: *Aspergillus nidulans*, *Aspergillus fumigatus*, *Emericella nidulans*, *Neurospora crassa*, *Saccharomyces cerevisiae, Schizosaccharomyces pombe* (Fig 2a). Analysis of these data allowed isolation of 445 non-redundant Expressed Sequence Tags (ESTs) with *E* value lower than 10^{-5}. Only eight of the 445 ESTs were represented by more than eight different cDNA clones. The two reciprocal subtracted libraries contained 197 and 275 potentially up- and down-regulated ESTs, respectively; 27 of them were shared by both libraries. The results obtained were available from our website at http://pagesperso.univ-brest.fr/~vasseur/Nevarez_et_al_2008/. The 197 potentially up-regulated ESTs were composed by 108 identified ESTs, 48 ESTs with hypothetical functions and 41 ESTs that appeared unknown in the databases. From the 275 ESTs contained in the down-regulated library, 195 matched with annotated sequences, 47 had hypothetical functions and 33 appeared to be unknown. Of all the isolated ESTs, 64 % showed similarity with annotated ORFs, 20 % encoded for hypothetical proteins and 16 % did not match any sequence in the public databases (Fig. 2b).

The analysis of both subtracted libraries showed a wide variety of ESTs encoding for proteins of different functional classes (Fig. 2c and 2d). Several ESTs coding for proteins often associated with cellular stress (heat shock proteins, trehalose-6-phosphate synthase, superoxide dismutase) appeared up-regulated after thermal stress (Fig. 2c). ESTs encoding other categories of proteins (metabolism, protein fate, transport proteins, etc.) showed relatively similar representation in both libraries. Some ESTs appeared down-regulated in *P. glabrum* after heat shock (Fig. 2d), as those encoding proteins involved in transcription (RNA polymerase, initiation factor, splicing factors, etc.) or implicated in protein synthesis (ribosomal proteins, translation initiation factors, elongation factors, etc.). Other ESTs isolated in each library coded for hypothetical or unknown proteins

cDNA microarray construction and RNA target preparation

The differential expression of ESTs isolated from SSH was analysed using custom-made cDNA microarrays. Amplified cDNAs isolated from up- and down-regulated subtracted libraries were spotted on cDNA microarrays. Each slide contained, in triplicate, the 913 subtracted cDNA corresponding to 445 different ESTs isolated from SSH. Many ESTs were represented by one to four different cDNA sequences and some were even represented by up to eight cDNAs. This redundancy was useful for evaluating the reproducibility of the expression results obtained for each EST.

Before target preparation, the quality of total RNAs extracted was first verified. Analysis of total RNA triplicates from C conditions revealed a convenient profile with three different and clearly defined peaks, corresponding to migration marker, 18S and 28S rRNAs. Similar results were obtained for total RNA triplicates extracted from S1, S2, S3 and S4 (data not shown). Analysis of total RNA samples from the more drastic S5 condition (47°C, 42

min) showed a migration marker peak and a large amount of very small RNA fragments indicating substantial total RNA degradation. We thus excluded the S5 stress condition from the central composite design.

Determination of housekeeping genes for validation of gene expression using RT-q-PCR

The final validation of the microarray results by qRT-PCR, requires previous selection of internal reference genes for qRT-PCR normalisation. Expression of the eight potential housekeeping genes selected (β-actin, adenosine kinase, 14-3-3 protein homolog, FAD dependent sulfhydryl oxidase, copper-fist DNA binding domain protein, ATP synthase gamma chain, Pre-mRNA splicing factor ini1 and S-phase kinase-associated protein 1A) was assessed under the different experimental conditions (C, S1, S2, S3, S4) with qRT-PCR. For each primer pair tested, PCR specificity was confirmed by melting curve analysis (data not shown). Expression results obtained for the eight genes tested were comparable, where Ct values were stable under C, S1, S2 conditions but substantially increased under S3 and S4 conditions (Fig. 3). Given these results, normalisation of qRT-PCR results could not be applied to S3 and S4 conditions since stable expression of internal control genes is required for each tested condition. As microarray data could not be validated for these thermal stress conditions, analysis was only done on S1 and S2 conditions. Beta-actin was selected as housekeeping gene for qRT-PCR normalisation, owing to its stable expression under C, S1, S2 conditions and its frequent use in the literature. ATP synthase gamma chain and adenosine kinase were also useful genes and were used to further confirm qRT-PCR normalisation (data not shown).

Microarray results for S1 and S2 thermal stress conditions

Based on the 913 cDNA sequences spotted in triplicate representing 445 different ESTs, the statistical analysis of results with a global ANOVA and LSD test indicated 35 and 49 significantly up- and down-regulated ESTs. Their S-to-C expression ratios corresponded to at least a +/-0.7 $\log_2$ (1.62-fold change). Expression of most of the isolated ESTs represented a *ca.* +/-2-fold change, but a few ESTs showed up to +3.5- and even a -5.0-fold change for up- and down-regulated ESTs, respectively.

Analysis of the microarray results revealed 49 highly significant down-regulated ESTs under thermal stress conditions S1 and S2 (Table 2). From this set, 26 ESTs encoded ribosomal proteins and accounted for 53% of the results. This large group also contained ESTs encoding proteins implicated in transcription (bZip transription factor CpscA, transcriptional activator HAA1, histone H4, etc.), RNA splicing (U6 small nuclear RNA associated protein, mago nashi protein homolog) and protein synthesis (translation elongation factors G2). A few proteases (glutamate carboxypeptidase-like protein, candidapepsin 2 precursor (aspartate protease)) and proteins involved in general metabolism (xanthine phophoribosyltransferase 1 and ornithine decarboxylase) were also observed. Few down-regulated ESTs also encoded miscellaneous proteins and few hypothetical or unknown proteins.

From the microarray results, 35 highly significant up-regulated ESTs were identified for thermal stress conditions S1 and S2 (Table 3). From this set, six and four ESTs encoded heat shock proteins (HSP 30, HSP 60, HSP 70, mitochondrial HSP 70, HSP 90, HSP 98/104) and proteins with antioxidant functions (superoxide dismutase, glutathione S-transferase 3,

cytochrome c oxidase polypeptide VIa, flavohemoprotein), respectively. The up-regulated EST set also included seven other ESTs coding for metabolism proteins (L-xylulose reductase, mannitol-1-phosphate-5-dehydrogenase, 2,3-diketo-5-methylthio-1-phosphopentane phosphatase, carboxylesterase, NADP-dependent alcohol dehydrogenase, trehalose-6-phosphate synthase). Up-regulated ESTs were also completed by few ESTs encoding proteases (aspergillopepsin A and vacuolar protease A) and other miscellaneous proteins. About 37% of identified up-regulated ESTs coded for hypothetical or unknown proteins.

Validation of microarray results with RT-q-PCR

The microarray results obtained for S1 and S2 conditions were validated by assessing the expression level of a set of 10 differentially expressed ESTs selected from the microarray analysis using qRT-PCR. Relative expression ratios of the 10 selected ESTs were normalised against β-actin gene expression and results obtained were comparable to results from the microarray (Table 4). The Bland–Altman plot (Fig. 4) carried out with values obtained for S1 and S2 conditions, showed that all of the $\log_2$–transformed microarray data and qRT-PCR data lay within the ''95 % confidence intervals'', delimited by a difference of 1. Moreover, more than 65% of the comparisons differed by less than 0.5. This similarity between both sets of data validate the microarray results for S1 and S2 conditions. Reliability of the data was further verified as similar results were also obtained by normalising qRT-PCR results with expression of the two other housekeeping genes selected: adenosine kinase and ATP synthase (data not shown).

Discussion

In the first part of the study, SSH was applied on *Penicillium glabrum* to isolate 913 cDNA sequences corresponding to 445 differentially expressed ESTs in response to thermal stress (i.e. 40°C, 120 min). SSH is frequently employed to isolate differentially expressed genes in response to varying nutritional or environmental conditions. SSH overcomes the problem of transcript abundance differences in the mRNA population by incorporating a normalisation step which equalises the relative quantity of the cDNA. It enhances the probability of identifying the increased expression of low-abundance transcripts (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996).

Many of the isolated ESTs encoded proteins with a wide variety of functions while some of them corresponded to hypothetical or unknown proteins. The large number of ESTs is remarkable compared to results obtained from similar studies on other fungi. For example, SSH was used to isolate specific transcripts in either yeast or mycelial forms of *Ophiostoma piceae* or *P. marneffei* and only revealed 50 and 43 genes, respectively (Dogra & Breuil, 2004; Liu *et al.*, 2007). Other studies conducted on the effects of different nutritional growth conditions, isolated 37 genes implicated in growth with penicillin-repressing or non-repressing carbon sources in *P. chrysogenum* (Castillo *et al.*, 2006). The large number of ESTs isolated here in *P. glabrum* under heat shock conditions (i.e. 40°C, 120 min) and their wide functional classification, could be explained by the fact that temperature is involved in general cellular mechanisms and influences many functional categories. It has been shown that two-thirds of the *Saccharomyces cerevisiae* genome is involved in response to environmental changes such as temperature, oxidation, nutriments, pH and osmolarity (Causton *et al.*, 2001).

Expression of ESTs contained in both subtracted libraries was further assessed, using microarrays to confirm their differential expression under thermal stress conditions. Custom cDNA microarray is a very convenient tool for studying subtracted libraries in details and are more appropriate than Northern blots or qRT-PCR methods for assessing the expression of a large number of ESTs. The combination of SSH and custom cDNA microarrays has been previously employed in *Gibberella zeae* to identify genes involved in sexual reproduction (mediated by ascospores) and implicated in disease occurring in cereal crops (Lee *et al.*, 2006). In our study, the custom microarray used in triplicates of the 913 subtracted cDNA sequences isolated corresponded to 445 different ESTs. Beyond just confirming the subtracted library approach, microarrays were also employed to analyse expression of these ESTs under five different thermal stress conditions (S1, S2, S3, S4 and S5).

Degradation total RNA observed in S5 conditions (47°C, 198min) leads us to eliminate this experiment from the study. This result may be correlated with a decrease of mycelium growth observed under these conditions: fungal dry weight decreased about 35% compared to dry weight in optimal growth conditions (unpublished data). Microarray analysis for the four other stress conditions, required final validation by qRT-PCR and prior selection of an internal reference gene for normalisation. As no housekeeping genes have yet been defined for *P. glabrum* or closely related fungal species, comparative studies and microarrays were used. Other than the seven potential housekeeping genes selected after microarray analysis, the β-actin gene was also chosen among other well-known internal reference genes such as β-tubulin, glyceraldehyde-3-phosphate dehydrogenase and 18S ribosomal RNA (Bustin, 2000; Livak & Schmittgen, 2001). Selection of β-actin was based on its frequent use in qRT-PCR normalisation in *Candida albicans* (Nailis *et al.*, 2006), *Phytophthora infestans* (Armstrong *et al.*, 2005) and *Neurospora crassa* (Mohsenzadeh *et al.*, 1998). The systematic increase in Ct values observed using qRT-PCR for every gene tested in S3 and S4 conditions

compared to C, S1 and S2 conditions in spite of the fact that mRNA quantity and quality was validated before RT. Variation in cDNA abundance can be partially explained by mRNA instability after exposure to high temperatures (Bond, 2006). For example, substantial decay of Hsp70 mRNA was reported in *S. cerevisiae* after a 3-h exposure to 48°C (Kapoor *et al.*, 1995). Messenger RNA decay primarily involves deadenylation or decapping (Tucker & Parker, 2000; Wilusz *et al.*, 2001). These processes probably affect mRNA stability and reverse transcription (Fleige & Pfaffl, 2006) without any change in the global quantity of mRNA. Given the increase in Ct values, housekeeping genes were not stable and microarray analyses were not possible for S3 and S4 conditions. This result was surprising since a preliminary study in *P. glabrum* only showed a slight decrease in growth under similar conditions (40°C, 120 min; unpublished data). Analysis of the quality of the total RNAs extracted from S3 and S4 conditions indicated that RNA integrity appeared conserved under both conditions. In addition, other thermal stress studies on fungi have been successfully conducted at temperatures close to 40°C: 37°C for *S. cerevisiae* (Gasch *et al.*, 2000; Causton *et al.*, 2001; Sakaki *et al.*, 2003), 39° for *Schizosaccharomyces pombe* (Chen *et al.*, 2003), 42 °C for *N. crassa* (Mohsenzadeh *et al.*, 1998), 45 °C for *C. albicans* (Zeuthen & Howard, 1989) and 50°C for *Aspergillus* (Bai *et al.*, 2003). Nevertheless, results obtained for S3 and S4 conditions clearly showed that defining a standard "housekeeping" gene can be difficult for experiments conducted under diverse conditions as reported by some authors (Schmittgen & Zakrajsek, 2000; Yan & Liou, 2006).

Given these results, both S3 and S4 conditions were excluded from the microarray analysis and results obtained for S1 and S2 were then confirmed using qRT-PCR on 10 genes as observed in the Bland-Altman plot (see Fig. 4). Statistical treatment of the microarray data, identified 35 significantly up-regulated ESTs and 49 significantly down-regulated. Some of

the down-regulated ESTs encoded proteases or general metabolism proteins. To our knowledge none of these genes have ever been described as being down-regulated in previous thermal stress studies performed on fungi. Another group of down-regulated ESTs encoded proteins implicated in transcription, RNA splicing and protein synthesis. Several studies carried out on *S. cerevisiae* exposed to thermal stress (25°C to 37°C) have also reported down-regulation of genes coding for histone H4 (Gasch *et al.*, 2000; Causton *et al.*, 2001; Sakaki *et al.*, 2003), translation elongation factor G2 (Sakaki *et al.*, 2003) and U6 small nuclear RNA associated protein (Gasch *et al.*, 2000). The group of ESTs involved in translation included a large set of 27 ESTs encoding ribosomal proteins. Down-regulation of these ESTs and repression of the corresponding protein synthesis have been frequently reported in the literature. In *S. cerevisiae*, it has been shown that ribosomal protein synthesis is repressed after exposure to 33°C for 90 min (Gorenstein & Warner, 1976), seemingly linked to a decrease of their corresponding mRNAs (Hereford & Rosbash, 1977) caused by inhibition of their transcription (Kim & Warner, 1983). Microarray studies at 25° to 37°C in *S. cerevisiae* and at 30° to 39°C in *Sc. pombe* (Gasch *et al.*, 2000; Causton *et al.*, 2001; Chen *et al.*, 2003; Sakaki *et al.*, 2003) also confirmed down-regulation of this genes in response to thermal stress. For example, in *S. cerevisiae* mRNAs have been reported to represent an important part of total poly(A)$^+$ mRNAs; repression of these genes may therefore allow energy to be dedicated to increased expression of genes involved in protective response (Jelinsky & Samson, 1999). This last group of ESTs encoding for ribosomal proteins, may contain some potential candidates for molecular markers of thermal stress in *P. glabrum*.

In addition, we identified 35 up-regulated ESTs that encoded proteases and some proteins implicated in metabolism. As for down-regulated ESTs, and to our knowledge, none of these genes have been previously reported in the literature on fungi, except trehalose-6-

phosphate synthase (tpsA), frequently described as being induced under heat shock conditions in yeasts (Gasch *et al.*, 2000; Causton *et al.*, 2001; Chen *et al.*, 2003; Sakaki *et al.*, 2003). Trehalose has been described to act as a protectant against various environmental stresses and has been showed to stabilize cellular structures under stress conditions (Tereshina, 2005). This disaccharide has been previously described in *S. cerevisae* to play a role in the acquisition of stress tolerance (De Virgilio *et al.*, 1994; Hottiger *et al.*, 1994; Singer & Lindquist, 1998). For *P. glabrum*, although it has never been reported in the literature, an accumulation of trehalose concurs with our preliminary results (unpublished data).

A group of up-regulated ESTs encoding HSPs was also identified in our study. These highly conserved proteins are certainly the most investigated element of the thermal stress response and increase in expression of the corresponding genes has been frequently observed in thermal stress studies (Lindquist, 1986). These proteins are classified into several families according to their molecular mass and, despite their different roles and proprieties, most HSPs have been shown to generally act as molecular chaperones. They are mainly involved in folding, assembly, regulation, degradation of other proteins and play an important role in thermotolerance (Craig, 1985; Feder & Hofmann, 1999).

Exposure to elevated temperatures has been previously reported to generate reactive oxygen species (ROS) responsible for oxidative damage in yeasts and filamentous fungi (Davidson *et al.*, 1996; Noventa-Jordao *et al.*, 1999; Bai *et al.*, 2003). As observed in our results, antioxidant systems appear to be extremely important for detoxifying cells. The synthesis of enzymes such as superoxide dismutase, glutathione S transferase, cytochrome c oxidase VIa, may serve to reduce the level of ROS in cells (Choi *et al.*, 1998; Marques *et al.*, 2004). In *S. cerevisiae*, it was shown that the synthesis of superoxide dismutase is enhanced in thermal stress conditions (Piper, 1995) and plays a role in resistance to heat shock from 30°C to 50°C (Davidson *et al.*, 1996). Microarray studies on the yeasts *S. cerevisiae* and *Sc. pombe*

have also confirmed induction of genes encoding superoxide dismutase, cytosolic glutathione S-transferase and cytochrome c oxidase popypeptide VIb (which has similarity to cytochrome c oxidase popypeptide VIa gene isolated in this study) (Gasch *et al.*, 2000; Causton *et al.*, 2001; Chen *et al.*, 2003). These ESTs coding for antioxidant proteins also appeared to be induced in heat shocked *P. glabrum* and could be potential molecular markers of thermal stress.

Considering these results, the present study provides a preliminary basis for the genetic regulation of thermal stress response in *P. glabrum*. Temperature is one of the most important factors for fungal growth and as others may represent potential sources of stress that can directly affect the physiology of fungi (growth, conidiation, synthesis of secondary metabolites, etc.). The transcriptional analysis performed here combined efficient and complementary molecular techniques making it possible to isolate 84 heat-regulated ESTs having a wide variety of functions and to assess their expression under thermal stress conditions. Some of these ESTs have already been associated with different stress-related responses as they encode heat shock proteins, trehalose-6-phosphate synthase, superoxide dismutase, ribosomal proteins or proteins involved in transcription/translation. In *P. glabrum*, they may be "common environmental stress response" genes and respond in a similar manner to many different environmental changes as those identified in studies on yeasts (Gasch *et al.*, 2000; Chen *et al.*, 2003). On the other hand, a large number of isolated ESTs encoded general metabolism proteins, proteases, miscellaneous proteins and hypothetical or unknown proteins. To our knowledge, a large majority of them have never been associated with fungal stress before and may represent new potential stress markers to characterise the physiological state of *P. glabrum* (stressed or unstressed). Nevertheless as reported by some authors, caution should be taken when analysing microarray results as mRNA stability and decay induced by

stress treatments may vary and have some influence gene expression profiling (Monje-Casas *et al.*, 2004; Michan *et al.*, 2005). Further investigations may study more precisely the stability of each mRNA and investigate the response of the different potential markers isolated.

Acknowledgements

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Tables :

Table 1:

(a) Primer sequences for the set of eight potential housekeeping genes. (b) Primer sequences for the set of 10 genes selected for validating the microarray results with qRT-PCR

a

EST	Primer	Primer sequence 5'->3'
β-actin	β-actin-F	ACGTTGTCCCCATCTACGAA
	β-actin-R	GCTCAGCCAGGATCTTCATC
Adenosine Kinase	AK-F	CTGGAGGACTCCGTTGACAT
	AK-R	TCCCAGAGTGGAAAATCCTG
14-3-3 protein homolog	14-3-3-F	CCGAGCGCTATGAAGAGATG
	14-3-3-R	ACTCCTCCTTCTGCTCGATG
FAD dependent sulfhydryl oxidase	FAD-F	AACCTCATTATGCGCCTCAC
	FAD-R	GGGCTTCCTGAAGCTTTTCT
Copper fist DNA binding domain protein	Copper fist-F	AGGTAGTCCGACCAATGACG
	Copper fist-R	GGCGATATTTCCGAAGTTGA
ATP synthase gamma chain	ATP synth-F	AACCTCATTATGCGCCTCAC
	ATP synth-R	GGGCTTCCTGAAGCTTTTCT
Pre-mRNA splicing factor ini1	Pre-mRNA-F	TACCCCTCCACTCCACAAAG
	Pre-mRNA-R	GGTACACGCCTTGAGAAGGA
S-phase kinase-associated protein 1A	S-Phase-F	ATGATGACAATCGTCGCAAG
	S-Phase-R	GGGGATTTGCCCTTAATCAT

b

EST	Primer	Primer sequence 5'->3'
Heat shock protein 70	Hsp 70-F	CCCGTCATTGAGGTTGAGTT
	Hsp 70-R	TGGGAGTCGTTGAAGTAGGC
Heat shock protein 98/104	Hsp 98-F	GAATGATTGGTGCACCTCCT
	Hsp 98-R	CCATCAGCTGCAAAAGAACA
Trehalose-6-phosphate synthase	TpsA-F	TGGGTGTCTTGCATTGTGAT
	TpsA-R	CTTTTCAGGGTCGATTCCAA
Import inner membrane translocase tim50	Tim50-F	AGGGTAGCCAGATCCCTGTT
	Tim50-R	AGACCTCGCCATCCATAGTG
NADP-dependant alcohol dehydrogenase	NADP Alcohol DH-F	TGACAGGCTTGATTTGGTGA
	NADP Alcohol DH-F	GGTGACCTGAAGGTTCCGTA
2,3-diketo-5-methylthio-1-phosphopentane phosphatase	2,3 phosphatase-F	TCAAGCCAGCACTGATAACG
	2,3 phosphatase-R	CGGTCTTTCCGTGAAGTCTC
Conserved hypothetical protein AN5480.2	AN5480.2-F	AGAATCTTCCAGCCGTTCAA
	AN5480.2-R	ATCATGACTCGCGGAGAAAG
Hypothetical protein EAL90164.1	EAL90164.1-F	CCTCCCAACCTCAAACTCAA
	EAL90164.1-R	GTCATCATCGGACTGGGACT
AAA family ATPase	AAA ATPase-F	GCGACACTCTCAGCAGTCAG
	AAA ATPase-R	CAAGGGTCAGGAGGACTTCA
Transcriptional activator HAA1	HAA1-F	TCGCATAAAGCGAAAAGGTC
	HAA1-R	TGTGAATTTGCGGTTGTGTT

Table 2:

List of ESTs isolated using microarrays as significantly down-regulated in thermal stress conditions S1 and S2.

Gene homolog	Accession number		Fold change		Significance
	Swiss-prot	Genbank	(S1-C)	(S2-C)	
<u>Ribosomal proteins:</u>					
40S ribosomal protein S2	P25443		-1,99	-1,87	***
40S ribosomal protein S5 a / b	O14277 / Q9P3T6		-2,20	-1,77	***
40S ribosomal protein S8 a / b	O14049 / Q9P7B2		-2,21	-1,69	***
40S ribosomal protein S9	P52810		-2,44	-1,87	***
40S ribosomal protein S11	P79013		-2,05	-1,65	***
40S ribosomal protein S12	O59936		-2,85	-2,20	***
40S ribosomal protein S16	Q42340		-2,39	-1,85	***
40S ribosomal protein S17	P27770		-2,12	-1,53	***
40S ribosomal protein S23	Q873W8		-2,56	-2,19	***
40S ribosomal protein S25	Q7SC06		-2,03	-1,68	***
60S ribosomal protein L2	Q75AP7		-1,68	-1,40	***
60S ribosomal protein L10a	Q7RZS0		-3,02	-2,42	***
60S ribosomal protein L11	Q758S7		-1,75	-1,76	***
60S ribosomal protein L14 a	P36105		-2,49	-1,83	***
60S ribosomal protein L15	O13418		-2,92	-2,21	***
60S ribosomal protein L18b	Q8TFH1		-2,17	-1,90	***
60S ribosomal protein L23	P04451		-2,50	-1,85	***
60S ribosomal protein L26b	Q9FJX2		-2,38	-1,92	***
60S ribosomal protein L30	Q7S7F1		-4,02	-2,27	***
60S ribosomal protein L34 b	P40525		-3,76	-2,76	***
60S ribosomal protein L35	Q8L805		-2,31	-1,65	***
60S ribosomal protein L38	Q9C2B9		-2,90	-2,15	***
60S ribosomal protein L44	P52809		-2,83	-2,25	***
60S ribosomal protein L5	O59953		-1,83	-1,46	***
60S ribosomal protein L8	O13672		-1,90	-1,55	***
Ribosome biogenesis protein RPF2	P36160		-1,72	-1,46	**
<u>Transcription and translation proteins:</u>					
Histone H4	P04914		-1,82	-1,34	***

Protein	Accession 1	Accession 2	Value 1	Value 2	Significance
Translation elongation factor G2		EAL91882.1	-1,85	-1,50	***
Translationally controlled tumor protein homolog	P35691		-2,06	-1,58	***
Transcriptional activator HAA1	Q12753		-2,77	-2,90	**
bZIP transcription factor CpcA		EAL89546.1	-1,93	-1,96	**
U6 snRNA-associated protein LSm3 (splicing)	Q9Y7M4		-1,92	-1,57	***
Mago nashi protein homolog (splicing)	P49030		-1,95	-1,64	*

Metabolism proteins:

Protein	Accession 1	Accession 2	Value 1	Value 2	Significance
Xanthine phosphoribosyltransferase 1	P47165		-1,85	-1,54	**
Ornithine decarboxylase	P27121		-2,58	-2,14	*

Proteases:

Protein	Accession 1	Accession 2	Value 1	Value 2	Significance
Candidapepsin 2 precursor (Aspartate protease)	P28871		-2,01	-1,97	***
Glutamate carboxypeptidase-like protein	Q9P6I2		-1,63	-1,37	*

Miscellaneous proteins:

Protein	Accession 1	Accession 2	Value 1	Value 2	Significance
AAA family ATPase		EAL86805.1	-1,73	-2,19	***
Guanine nucleotide-binding protein (protein G)	Q01369		-2,01	-1,53	***
Prefoldin subunit 6 (actin chaperone protein)		EAL92646.1	-2,18	-1,58	***

Hypothetical proteins / unknown proteins :

Protein	Accession 1	Accession 2	Value 1	Value 2	Significance
Hypothetical protein Afu1g07570		EAL88465.1	-1,64	-1,47	***
Hypothetical protein AN0409.2		XP_658013.1	-2,13	-1,81	***
Hypothetical protein AN5226.2		XP_662830.1	-2,21	-1,64	**
Hypothetical protein MG04362.4		XP_361917.1	-2,21	-2,07	*
Unknown protein F17			-1,94	-1,41	***
Unknown protein F26			-1,74	-1,49	***
Unknown protein F6			-2,84	-2,60	***
Unknown protein F9			-2,08	-1,77	***
Unknown protein R16			-3,70	-2,71	***

* Significant

** Highly significant

*** Very highly significant

Table 3:

List of ESTs isolated using microarrays as significantly up-regulated in thermal stress conditions S1 and S2.

Gene homolog	Accession number		Fold change		Significance
	Swiss-prot	Genbank	(S1-C)	(S2-C)	
Chaperones:					
Heat shock protein 30	P40920		+2,67	+2,51	***
Heat shock protein 60	O60008		+1,74	+1,43	***
Heat shock protein 70	Q92260		+2,98	+1,93	***
Heat shock protein 90	P40292		+3,55	+2,06	***
Heat shock protein 98 / 104	P31540		+2,28	+2,06	***
Mitochondrial Hsp70 chaperone (Ssc70)		EAL93290.1	+1,66	+1,35	**
Antioxidant proteins:					
Cytochrome c oxidase polypeptide VIa	P32799		+1,65	+1,52	**
Superoxide dismutase	Q9UQX0		+1,65	+1,62	***
Flavohemoprotein (Hemoglobin-like protein)		EAL84490.1	+1,69	+1,26	***
Glutathione S-transferase 3		AAX07321	+1,87	+1,60	***
Metabolism proteins:					
Trehalose-6-phosphate synthase tpsA	O59921		+2,36	+1,67	***
2,3-diketo-5-methylthio-1-phosphopentane phosphatase		EAL86676.1	+4,34	+2,33	***
Carboxylesterase		EAL92659.1	+2,14	+1,60	***
L-xylulose reductase	Q8NK50		+3,00	+1,92	***
Mannitol-1-phosphate 5-dehydrogenase	Q6AHH7		+3,22	+2,18	***
Plasma membrane ATPase (proton pump)	P24545		+1,69	+1,42	***
NADP-dependent alcohol dehydrogenase		EAL84214.1	+2,64	+1,77	***
Proteases:					
Aspergillopepsin A	Q06902		+1,68	+1,63	**
Vacuolar protease A	Q01294		+1,78	+1,63	***
Miscellaneous proteins:					
Metalloreductase, putative		EAL85757.1	+1,71	+1,60	**

Import inner membrane translocase subunit tim50	Q4WI16	+1,66	+1,66	**
Protein bli-3 (blue light-inducible protein)	EAL90736.1	+2,30	+2,05	**

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Hypothetical proteins / unknown proteins:

Integral membrane protein	EAL87419.1	+2,51	+2,02	***
Predicted protein	XP_322336.1	+1,71	+1,86	***
Conserved hypothetical protein	EAL90164.1	+3,58	+2,26	***
Hypothetical protein AN0677.2	XP_658281.1	+1,76	+1,46	**
Hypothetical protein AN5056.2	XP_662660.1	+2,42	+2,00	**
Hypothetical protein AN5480.2	XP_663084.1	+3,23	+1,99	***
Hypothetical protein AN5537.2	XP_663141.1	+2,12	+1,68	***
Hypothetical protein AN9515.2	XP_868897.1	+3,01	+1,87	***
Unknown protein F18		+1,77	+1,66	***
Unknown protein F23		+1,70	+1,78	**
Unknown protein R10		+2,57	+1,71	***
Unknown protein R29		+1,96	+1,60	**
Unknown protein R36		+2,93	+1,91	**

* Significant

** Highly significant

*** Very highly significant

Table 4:

Microarray expression ratios and relative expression results obtained with qRT-PCR in S1 and S2 conditions for the 10 selected ESTs. β-actin gene expression was used for normalisation of qRT-PCR results.

| EST | Accession number | S1-C | | S2-C | |
| | | Relative expression | | Relative expression | |
		Microarrays	qRT-PCR	Microarrays	qRT-PCR
Heat shock protein 70	Q92260	+2,98	+4,41	+1,93	+2,71
Heat shock protein 98 / 104	P31540	+2,28	+3,34	+2,06	+3,68
Trehalose-6-phosphate synthase	O59921	+2,36	+4,22	+1,67	+2,07
Import inner membrane translocase subunit tim50	Q4WI16	+1,66	+1,87	+1,66	+1,98
NADP-dependent alcohol deshydrogenase	EAL84214.1	+2,64	+4,17	+1,77	+2,49
2,3-diketo-5-methylthio-1-phosphopentane phosphatase	EAL86676.1	+4,34	+3,80	+2,33	+2,17
Hypothetical protein AN5480.2	XP_663084.1	+3,23	+2,19	+1,99	+1,24
Conserved hypothetical protein EAL90164.1	EAL90164.1	+3,58	+3,84	+2,26	+1,80
AAA family ATPase	EAL86805.1	-1,73	-2,25	-2,19	-2,81
Transcriptional activator HAA1	Q12753	-2,77	-2,67	-2,90	-2,91

Figure legends:

Fig. 1. The central composite design used to investigate transcriptional effect of thermal stress in *Penicillium glabrum*.

Fig. 2. Transcriptional analysis of *Penicillium glabrum* under thermal stress (40°C, 120 min) with SSH. (a) Global distribution of BlastX best hits of up- and down-regulated subtracted libraries among organisms; (b) Global view of proteins encoded by ESTs isolated in both up- and down-regulated subtracted libraries. (c) Functional classification of proteins encoded by ESTs isolated in the up-regulated library and (d) the down-regulated subtracted library.

Fig. 3. Results obtained with qRT-PCR (Ct) for potential housekeeping genes in control (C) and thermal stress conditions (S1, S2 , S3 and S4). □ ATP synthase gamma chain , ▲ β-actin, ♦ adenosine kinase, ○ S-phase kinase-associated protein 1A, ■ 14-3-3 protein homolog, △ copper-fist DNA binding domain protein, ● FAD dependent sulfhydryl oxidase, ◊ pre-mRNA splicing factor ini1.

Fig. 4. Bland–Altman plot showing the agreement between expression ratios obtained in S1 and S2 conditions, with qRT-PCR and microarray analysis for the 10 selected genes.