The Perigord black truffle responds to cold temperature with an extensive reprogramming of its transcriptional activity

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Abstract

The *Tuber melanosporum* genome has been analysed with the aim of identifying and characterizing the genes involved in the environmental stress response. A whole genome array (7496 genes/probe) was used to verify the fungal transcriptional profiling upon a cold temperature period (7 days at 4 °C). A total of 423 genes resulted to be differentially expressed in a significant manner (>2.5-fold; *p*-value < 0.05) in the mycelia exposed to cold, compared to the control ones: 187 of these genes were up-regulated, while 236 were down-regulated. Sixty-six and fifty-one percent, respectively, of the up- or down-regulated transcripts had no KOG classification and were clustered as unclassified proteins, which was the most abundant category in the both up- and down-regulated genes. A gene subset, containing a range of biological functions, was chosen to validate the microarray experiment through quantitative real time PCR (qRT-PCR). The analysis confirmed the array data for 16 out of 22 of the considered genes, confirming that a cold temperature period influences the truffle global gene expression. The expressed genes, which mostly resulted to be genes for heat shock proteins (HSPs) and genes involved in cell wall and lipid metabolism, could be involved in mechanisms, which are responsible for fungal adaptation. Since truffle ascomata develop during the winter period, we hypothesize that these differentially expressed genes may help the truffle to adapt to low temperatures and/or perceive environmental signals that regulate the fructification.

Keywords

- *Tuber melanosporum*;
- Environmental stress response genes;
- Whole genome microarray;
- Cold temperature;
- qRT-PCR

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1. Introduction

Free-living fungi often encounter different kinds of environmental stresses, including changes in temperature, osmolarity, pH, humidity, O_2 and nutrients availability, exposure to toxins, UV or heavy metals, and competition with other organisms (Gasch, 2007). Sudden changes in the external conditions can have a direct impact on the homeostasis and normal physiology of fungi, which have evolved elaborate mechanisms to check the environment and quickly activate adaptation systems (Gasch, 2007). Environmental factors can therefore be fundamental for morphogenetic changes. The role of UV/blue light has been reported to be important in *Neurospora crassa* in the maintenance and resetting of the circadian clock, in the carotenoid biosynthesis, in the asexual conidiation and in the sexual cycle, where light controls the formation of protoperithecia and the direction of ascospores release (Chen et al., 2009). Temperature is another one of the most important environmental parameters that can affect the growth and survival of microorganisms (Deegenaars and Watson, 1998). Murata et al. (2006) have studied the gene expression in *Saccharomyces cerevisiae* in the presence of a cold shock and have verified a transcriptional genome-wide response. The main regulated gene categories were related to energy, metabolism, cell rescue, defense and virulence, while the repressed genes on exposure to 4 °C were related to protein synthesis, binding, activity regulation and protein fate. Heat shock proteins (HSPs) were also regulated and their role has been suggested to be involved in the revitalization of enzyme activity. In general, HSPs are induced in various stress conditions, such as oxidative stress, high pressure and heat shock, and they play an important role in protein folding process, when proteins are denatured during changes in the environmental conditions (Murata et al., 2006). Gocheva et al. (2009), through different biochemical assays, demonstrated that growth at low temperatures (downshift from an optimal temperature to 15 °C and 6 °C) clearly induced oxidative stress events in filamentous fungi (*Penicillium* sp.); these events were identified by an enhanced level of oxidative damaged proteins, the accumulation of reserve carbohydrates and an increased activity of antioxidant enzyme defense (superoxide dismutase, catalase). Although several fungal genome projects (304 genome projects are listed in http://www.genomesonline.org), which allow careful comparisons of fungi that differ according to their phylogenetic relationships and life styles, have been launched (Hertz-Fowler and Pain, 2007), to our knowledge, no reports are available on the global responses to cold temperature in filamentous fungi.

The capacity of many fungi to adapt to cold temperatures can have an impact on their growth and distribution, with ecological consequences on the symbiotic/pathogenic relationships and on nutrient exploitation (Tibbett et al., 2002). Cold temperatures can in fact be considered as a natural environment parameter, which may affect the soil fungal life cycle; the ability to survive during winter is a key factor for the ecological success of ectomycorrhizal fungi, unlike arbuscular mycorrhizal (AM) fungi, which are sensitive to cold (Kytöviita, 2005). It has been reported that *Suillus luteus, Suillus variegatus, Laccaria laccata,* and *Hebeloma* sp. show a high capacity to tolerate low temperatures (between +5 °C and −48 °C) in pure cultures (Lehto et al., 2008). The ability of ectomycorrhizas to survive winter months in the field has already been reported (Tibbett et al., 2002). Truffles, the ectomycorrhizal ascomycetes, are also subjected to temperature fluctuations. For example, *Tuber melanosporum* mycelium grows in the soil, colonizes the roots of its host and establishes symbiosis during the spring. Extramatrical hyphae then aggregate to form the fruiting body initials, which develop and mature in the next winter, thus concluding their life cycle (Martin et al., 2010). In addition to a temperature change, other abiotic factors (soil composition and weather, such as rain or sunshine) and biotic factors are listed as determinants that enhance or inhibit ascocarp formation (Ceruti et al., 2003). However, at the moment, limited information exists concerning the molecular mechanisms that control these physiological/morphological changes, which result as a response to variations in temperature. A gene identified as coding for a dehydrin (DHN)-like protein was one of the most expressed genes

during *Tuber borchii* fruiting body maturation (Abbà et al., 2006); it showed high expression in the presence of cold conditions and osmotic stress. It was hypothesized that the up-regulation of TbDHN1 in fruiting bodies, compared to vegetative mycelium, is probably closely linked to the exposure of fruiting bodies to low temperatures during the autumn/winter season in respect to the mycelium growth at 24 °C (Abbà et al., 2006).

Taking advantage of the availability of *T. melanosporum* genome sequencing (Martin et al., 2010) and a whole genome microarray, we tested the hypothesis, whether the transition from a growth temperature of 24–25 °C to a cold one (4 °C) is perceived by the filamentous fungus at a transcriptomic level and whether this change acts as an environmental stimulus that might directly or indirectly elicit ascocarp development. The specific objectives were: (i) to identify and characterize the genes involved in environmental stress responses, and (ii) to validate their expression in the presence of a cold temperature (7 days at 4 °C), using transcriptional analysis of whole genome arrays and qRT-PCR.

2. Materials and methods

2.1. *In silico* functional annotation

In order to find stress-related sequences, the key word "HSP, DHN, stress" were inserted into the mycorweb website (http://mycor.nancy.inra.fr/), which contains the predicted gene models. The gene predictions, obtained by Eugene, were validated considering the model, the homologous gene structure of other Ascomycetes and the sequenced EST.

Using the Artemis software (version 11 http://www.sanger.ac.uk/Software/Artemis/), the presence of the start and stop codon and of the splicing sites of each gene was checked.

Further information about the name and structure of the gene was obtained using BLASTp (basic local alignment search tool) (Altschul et al., 1997) on NCBI (http://www.ncbi.nlm.nih.gov/) and EMBL (http://www.ebi.ac.uk/Tools/blastall/index.html) and against five sequenced Ascomycetes (*S. cerevisiae, N. crassa, Magnaporthe grisea, Aspergillus, Botrytis cinerea*). In some cases, the predicted models were modified, again using the Artemis software.

2.2. *T. melanosporum* cultures

The *T. melanosporum* strain Mel28 (Martin et al., 2010) was used in all the experiments. The fungus was inoculated in flasks containing 1% liquid malt. Each flask contained 50 ml of the 1% malt medium and the cultures were kept in a dark room at 25 °C. After a month, some flasks were moved to a 4 °C environment for 1 week. The mycelium was then collected from the treated (from 25 °C to 4 °C) and the untreated (25 °C) cultures, dried on paper and frozen in liquid nitrogen for the RNA extraction.

2.3. Morphological analysis

Morphological observations were performed on the mycelia grown in the pure culture (25 °C and 4 °C). The fungal cell wall was stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin (FITC-WGA; 10 mg/ml), while the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The stained sections were observed using a Leica TCS SP2 confocal microscope. An excitation wavelength of 488 nm and an emission window of 500–520 nm were

used to visualize the FITC; a 405 nm light was used to excite the DAPI, whose fluorescence was recorded between 430 and 500 nm.

2.4. RNA extraction

The tubes containing the frozen mycelium were put in a freeze dry machine overnight at −65 °C. The total RNA was extracted for microarray analysis using a LiCl method (Viotti et al., 1982). A clean up and a DNase treatment were conducted, following the RNeasy mini kit procedure (Qiagen). The RNA was resuspended in DEPC (diethylpyrocarbonate) water (30 μl). The RNA was checked using NanoDrop (Celbio) for the quantity and Experion (BIORAD) for the quality, and it was then freeze dried and suspended in DEPC water (12 μl).

2.5. Retrotranscription and amplification of cDNA

Five hundred nanogram of total RNA were used to synthesize the first strand of cDNA, following the SMART PCR cDNA synthesis kit procedure (Clontech) for each biological replica. After this synthesis, the best number of cycles was identified to amplify the cDNA, according to the SMART protocol. Each sample was amplified twice and purified, using a PCR purification kit (Qiagen).

The concentration was measured by the spectrophotometer, using a 1:10 dilution and then each product was checked in 1.2% TBE (Tris borate EDTA) gel. The replicates were joined together and then freeze dried in order to obtain a 250–220 ng/μl concentration.

The samples were sent to NimbleGen (Madison, WI) to be labelled and used as probes for the whole genome microarray.

2.6. Whole genome microarray

The *T. melanosporum* whole genome expression array was built by NimbleGen Systems Limited (Madison, WI). It contained five independent, non-identical, 60-mer probes per gene model. A total of 7496 annotated protein-coding gene models, 5736 TE (transposable element) sequences, 3913 randomly designed 60-mer control probes (which can be used to estimate background hybridization) and labeling controls were included in the oligoarray. The sequences used for the oligonucleotide design were taken from an early draft of the gene catalogue, which contained several TE families. Technical duplicates were included in the array for 1876 gene models (see GEO platform GPL8982) Reference can be made to Martin et al. (2010) for a detailed description. The complete expression dataset is available (series number GSE21626) from the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/).

2.7. Data analysis

After the single dye labeling and hybridization procedures, the data acquisition, background correction and normalization were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland), according to their standard protocol. The average expression levels were calculated for each gene from the independent probes and were used for further analysis. The raw array data were filtered for non-specific probes (a probe was considered non-specific if it shared more than 90% homology with a different gene model from the gene model it was made for) and renormalized using ARRAYSTAR software (DNASTAR). A Student *t*-test with FDR (Benjamini-Hochberg) multiple testing corrections was applied to the data, using ARRAYSTAR software (DNASTAR). The KOG definition of the corresponding gene models was used for the functional classification of the data.

2.8. RNA extraction, cDNA synthesis and quantitative realtime PCR

The *T. melanosporum* strain Mel28 cultures (two biological replicates for both the control and for the stress condition), described in the section above, were used to extract RNA for the post array validation.

RNA was extracted using the 'pine tree – method' (Chang et al., 1993). The genomic DNA was removed using the Turbo DNA-free™ reagent (Ambion, Austin, TX, USA), according to the manufacturer's instructions. The absence of genomic DNA was verified through retrotranscription PCR (RT-PCR, OneStep RT-PCR, Qiagen) with a intron-specific primer for the *Pda2* gene, coding for putative chitin/polysaccharide deacetylase (Tmelpda2 f and Tmelpda2 r. See the sequences in Table 1).

Table 1.

Primer list used in the qRT-PCR.

Name	Sequence 5′–3′
Tmelpp02 f	GCACGATTGATCACCAATGG
Tmelpp02 r	AGAGTCCACGTAGATTCGGA
Tmelcyp5aj3 f	ATCATTCAAGAAGTTCTCAGACTC
Tmelcyp5aj3 r	TCCTTTGCGAATGGAAATAGG
Tmelpir3 f	GACAGTTCCAGTTTGATGGACC
Tmelpir3 r	GATATTCTCGAAGGTACCGCTC
Tmelnira2 f	TGGATGGATGTACTCTGGACG
Tmelnira2 r	TACGGACCACATCTTGTCGA
Tmelnira1 f	GCAAGTCAAAGTGTGATGGG
Tmelnira1 r	GGATCGTAGACACATTCGGT
Tmelpda2 f	GGTCAGGCTTTGGACGATTC
Tmelpda2 r	GTGGGTGTAGGAGTGCAGAG
Tmeln.a. f	ATTCCACCGTGTTAAGGCTC
Tmeln.a. r	GCACCGAAACCAAATGTTGTC
Tmelhsp9_1 f	CTCCAGAGCGACCACCACAA
Tmelhsp9_1 r	AACACCGAGGGCGTGCTTAG
Tmelhsp9_2 f	TGTCTGATTTCGGAAGAAAGTC
Tmelhsp9_2 r	GGTTCTATCGTAGGCACCTG
Tmelhspa12a_1 f	CTTTCAAGGAGGGAAGTGAAGG
Tmelhspa12a_1 r	CGAATCCACCCACTAGAAGGA
Tmelhsp30_2 f	ACACCAGCAGCCTGTGCATC
Tmelhsp30_2 r	TAATTCCCGCGCTCCGCTTC
Tmeltef1 f	CGTTGCTATCAACAAGATGG
Tmeltef1 r	GTTGAAACCAGAGATAGGGA
TmelDHN f	CCTGATGAACAAATTGGACC
TmelDHN r	CGGCAGTATAAGTCTTATCAG
Tmelhsp60 f	GGTTGTTGGGAAACTTACTG

Name	Sequence 5′–3′
Tmelhsp60 r	ATCATGTTGACATATTCGCC
Tmelcyp1 f	AGGTGGTTCTTCGATTTATGGC
Tmelcyp1 r	GCAAGCGTGATAAAGAATTGAGAC
Tmelhsp90 f	TCTCAAGGAGGTTCTCGGTG
Tmelhsp90 r	CATCGAAGTATCACGCAGAGC
Tmelmdj1 f	GTCAGAGTGGATTTCGCGAG
Tmelmdj1 r	TGTTTCAACCTCAATGTTATCACC
Tmelfes f	AGTCTCAGGCCGTCATGCTC
Tmelfes r	GCGCATAAACCGCCTTGGTC
Tmelhyp f	CAGCGGCACAGCAGTATTTC
Tmelhyp r	GGTTCCCTATGGCGAGTTTG
Tmelpda3 f	TTGGTGCGGAGACACCCTTG
Tmelpda3 r	TGTAGGCGTGGGTGTTCCAG
Tmelzrc1 f	TGGTTGGTGAGGAAAGCG
Tmelzrc1 r	GTTTGCGGCCAACAATGTC
Tmelsodc f	GTGCGAATGGGATTGTGG
Tmelsodc r	GTGCTCCAATCAACCCTCC
Tmelpth11rel18 f	ATCTACGAATTGGTCCACCG
Tmelpth11rel18 r	GCCGAGGTTTAGGGTTGAG

Five hundred ng of total RNA was used for each sample to synthesize the cDNA, according to the SuperScriptII® Reverse Transcriptase (Invitrogen) procedure. qRT-PCR primers were designed using PerlPrimer, a free, open-source GUI application (http://perlprimer.sourceforge.net/). Before qRT-PCR, all the primers were tested on genomic DNA and cDNA for amplification purposes.

Quantitative RT-PCR was carried out with StepOne apparatus (Applied Biosystem). Each PCR reaction was conducted on a total volume of 20 µl, containing 1 µl diluted cDNA (166,6 ng), 10 µl SYBR Green Reaction Mix and 2 µl of each primer (3 µM) using a 48-well plate. The used primers are listed in Table 1. The following PCR programme, which includes the calculation of a melting curve, was used: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 for 1 min, and 95 °C for 15 s. All the reactions were performed for two biological and three technical replicates. The baseline range and *C*T values were automatically calculated using the StepOne software. In order to compare data from different PCR runs or cDNA samples, the *C*T values of all the genes were normalized to the *C*T value of *Tmeltef1-beta*.

The candidate gene expression was normalized to that of *Tmeltef1-beta* by subtracting the *C*T value of *Tmeltef1-beta* from the *C*T value of the candidate gene resulting in Δ*C*T. Since the slopes of the different samples and replicates in the exponential phase were the same, the expression ratios were calculated without PCR efficiency correction from equation $2^{-\Delta\Delta CT}$. ΔΔ*C*T represents the Δ*C*Tsample – Δ*C*Tcontrol. Statistical analyses were carried out using Rest2008, version 2.0.7, considering 0.05 as the *p*-value.

3. Results

3.1. Environmental stress-responsive genes in the *T. melanosporum* genome

The availability of the *T. melanosporum* genome sequence allowed an *in silico* annotation to be made. The analysis showed that the typical families of environmental stress response, such as those described by Borkovitch et al. (2004) in the *N. crassa* genome, are all present in the *T. melanosporum* genome. Seventy-four genes were annotated and *in silico* validated; among these 15 gene models were edited. Sixty-four of them were assigned to 16 gene families, identified through a Pfam search (http://pfam.sanger.ac.uk/) (for more details see (Martin et al., 2010) and the Supplemental material Table 1). The lack of members in Pam16, Hsp20, Hsp9/12, Cpn10, and Usp is in agreement with other genomes (*Aspergillus* sp., *N. crassa, B. cinerea and Neosartorya fischeri*). The validated genes in the Fes and Grpe families are also in line with *N. crassa* and *B. cinerea*. The Clp, DNAJ, FKBP and cyclophilin families seem to be underrepresented compared to the other Ascomycete genomes. These data, however, could be explained by the presence of non validated gene models.

3.2. Morphology of the *T. melanosporum* mycelium at 25 °C and 4 °C

As a preliminary step morphological observations were performed to investigate the responses of the black truffle to a temperature change (from 25 °C to 4 °C), using confocal microscopy applied to living hyphae sampled from the two growing conditions. No evident differences were revealed (data not shown).

3.3. Transcriptomic profile of the *T. melanosporum* mycelium at 25 °C *vs.* 4 °C through cDNA whole genome microarray

In order to characterize the *T. melanosporum* transcriptome in the presence of a temperature change (7 days at 4 °C, after growth at 25 °C), we performed a microarray experiment, based on a NimbleGen (Madison, WI) chip with three biological replicates for both the control and the cold condition.

Among the 7496 genes present in the chip, 423 were found to be differentially expressed with a significant *p*-value (<0.05) after 7 days of exposure at 4 °C. One hundred eighty-seven genes were up-regulated (>2.5-fold); 236 genes were down-regulated (>2.5-fold) (Supplemental material Table 2). After 7 days of exposure at 4 °C, the up- and down-regulated genes were classified into functional categories according to KOG. Sixty-six and fifty-one percent, respectively, of the up- or down-regulated transcripts had no KOG classification and were clustered as unclassified proteins, the most abundant category in both the up- and down-regulated genes. However, in some cases, through Blast similarity, it was possible to give a definition and a putative function. *TmelDHN1*, which was the most up-regulated gene in the array, was also found among these latter proteins (unclassified in the KOG classification). The up-regulated and down-regulated gene categories are reported in Table 2.

3.4. Validation of a subset of differentially expressed genes

Among the differentially regulated genes identified by the NimbleGen array (Table 2), 22 were selected for validation through qRT-PCR on the basis of their biological significance (refolding, maintenance of cell wall and membrane integrity, as well as homeostasis), considering both the genes with a KOG classification and those belonging to unclassified proteins, but with a Blast

similarity (Table 1). These mostly included members of gene families listed as environmental stress-responsive genes: HSPs such as *Tmelhsp60, Tmelhsp90, Tmelhsp30, Tmelhspa12a, Tmelhsp9_1, Tmelhsp9_2,* a Hsp70 nucleotide exchange factor FES1 (*Tmelfes1*), an uncharacterized protein C1711.08, a DnaJ homolog 1, a mitochondrial precursor (*Tmelmdj1),* a peptidyl-prolyl cis–trans isomerase-like 1 (*Tmelcyp1),* a hypothetical protein and the dehydrin (*TmelDHN1)*. Genes involved in other biological processes were also considered: a superoxide dismutase C (*Tmelsodc*), a candidate Zrc1 (vacuolar zinc transporter, *Tmelzrc1*), two putative chitin/polysaccharide deacetylases (*Tmelpda2*, *Tmelpda3*), a cell wall-related protein (*Tmelpir3*), a linoleate diol synthase (*Tmelpp02*), a cytochrome P450 52A13 (*Tmelcyp5aj3*), two nitrate regulatory proteins (*TmelNIRA1*, *TmelNIRA2*) and a probable G-protein coupled receptor, Pth11 related (*Tmelpth11rel18*).

Table 2.

Expression changes after 7 days at 4 °C.

	Total ORFs[a]	**4 °C**	**(%)**[c]
(a) Greater than 2.5-fold up-regulated gene		187[b]	
General function prediction only	660	14	2.1
Signal transduction mechanisms	482	9	1.9
Cytoskeleton	450	7	1.6
Transcription	385	5	1.3
Function unknown	197	5	2.5
RNA processing and modification	317	5	1.6
Replication, recombination and repair	187	5	2.7
Post-translational modification, protein turnover, chaperones	480	4	0.8
Secondary metabolite biosynthesis, transport and catabolism	136	4	2.9
Lipid transport and metabolism	219	3	1.4
Amino acid transport and metabolism	207	3	1.4
Inorganic ion transport and metabolism	117	2	1.7
Intracellular trafficking, secretion, and vesicular transport	365	2	0.5
Cell wall/membrane/envelope biogenesis	80	2	2.5
Energy production and conversion	248	2	0.8
Carbohydrate transport and metabolism	372	1	0.3
Defense mechanisms	41	0	0.0
Nuclear structure	46	0	0.0
Extracellular structures	26	0	0.0
Cell motility	4	0	0.0
Coenzyme transport and metabolism	87	0	0.0
Nucleotide transport and metabolism	80	0	0.0
Translation, ribosomal structure and biogenesis	352	0	0.0
Chromatin structure and dynamics	101	0	0.0
Cell-cycle control, cell division, chromosome partitioning	184	0	0.0
Unclassified proteins	3330	124	3.7
(b) Greater than 2.5-fold down-regulated gene		236[b]	

	Total ORFs[a]	4 °C	(%)[c]
General function prediction only	660	17	2.6
Signal transduction mechanisms	482	5	1.0
Cytoskeleton	450	11	2.4
Transcription	385	5	1.3
Function unknown	197	7	3.6
RNA processing and modification	317	7	2.2
Replication, recombination and repair	187	0	0.0
Post-translational modification, protein turnover, chaperones	480	7	1.5
Secondary metabolite biosynthesis, transport and catabolism	136	8	5.9
Lipid transport and metabolism	219	5	2.3
Amino acid transport and metabolism	207	9	4.3
Inorganic ion transport and metabolism	117	2	1.7
Intracellular trafficking, secretion, and vesicular transport	365	5	1.4
Cell wall/membrane/envelope biogenesis	80	2	2.5
Energy production and conversion	248	7	2.8
Carbohydrate transport and metabolism	372	5	1.3
Defense mechanisms	41	3	7.3
Nuclear structure	46	1	2.2
Extracellular structures	26	2	7.7
Cell motility	4	0	0.0
Coenzyme transport and metabolism	87	3	3.4
Nucleotide transport and metabolism	80	2	2.5
Translation, ribosomal structure and biogenesis	352	3	0.9
Chromatin structure and dynamics	101	1	1.0
Cell-cycle control, cell division, chromosome partitioning	184	4	2.2
Unclassified proteins	3330	122	3.7

a The total ORFs represent the total number of classified genes in each category according to the KOG definition.
b The KOG ID of a gene can be assigned to more then one KOG category.
c The percentage indicates the number of up- or down-regulated genes in proportion to the total ORFs.

The differential expression (cold stress vs. control condition) was confirmed for 16 genes, using RNA from two independent biological replicates. The levels of up- and down-regulation obtained in the array and in qRT-PCR were listed in Table 3. The up-regulated genes were: *TmelDHN1, Tmelhspa12a, Tmelhsp30, Tmelhsp9_1, Tmelhsp9_2, Tmelfes1, Tmelzrc1, Tmelpda2, Tmelpda3, Tmelpir3, Tmelcyp5aj3, TmelNIRA2,* and *Tmelpth11rel1,* while *Tmelhsp90* and an uncharacterized protein, C1711.08, were found to be down-regulated.

Table 3.

Expression values in the qRT-PCR and in the microarray analysis.

Gene Mode ID	Gene	Fold qRT-PCR	Fold array
GSTUMT00002556001	TmelDHN1	42.22	68.20
GSTUMT00001960001	Tmelhspa12a_1	4.906	2.45
GSTUMT00009341001	Tmelhsp60**	0.85	0.5
GSTUMT00008756001	Tmelhsp30_2	13.57	3.74
GSTUMT00004694001	Tmelhsp9_2	5.921	5.67
GSTUMT00012114001	Tmelhsp90	0.176	0.48
GSTUMT00012030001	Uncharacterized protein	0.141	0.24
GSTUMT00005334001	Tmelcyp1**	1.40	0.52
GSTUMT00006401001	Tmelmdj1	0.846	0.43
GSTUMT00000236001	Tmelhsp9_1	104.459	5.42
GSTUMT00003032001	Tmelpda2	14.154	3.66
GSTUMT00004210001	Tmelpda3	10.625	2.11
GSTUMT00006891001	Tmelpp02	86.506	10.42
GSTUMT00002786001	Tmelpir3	10.67	9.10
GSTUMT00001990001	Hypothetical protein	1.71	0.54
GSTUMT00004620001	Tmelcyp5aj3	92.90	4.29
GSTUMT00003209001	TmelNIRA2	98.765	32.23
GSTUMT00002283001	TmelNIRA1	5.599	0.45
GSTUMT00006161001	Tmelfes1	2.80	1.35
GSTUMT00006222001	Tmelsodc	0.35	2.41
GSTUMT00007113001	Tmelzrc1	2.82	2
GSTUMT00004274001	Tmelpth11rel18	29.957	2.82

The genes whose differences have no statistical significance are indicated with **; these genes, together with the underlined ones, did not validate the array results. Among the not confirmed genes, no highly regulated genes are present in the arrays. Genes only slightly regulated but with interesting function have been also selected for validation.

4. Discussion

4.1. Environmental stress-responsive genes are present in the truffle genome and respond to cold treatments

The sequencing of the *T. melanosporum* genome has revealed many characteristic features, including the high genome size and the low number of protein-coding genes (Martin et al., 2010). In spite of this, all the gene families listed as environmental stress-responsive families in the sequenced Ascomycetes, which always possess a higher gene number, like *N. crassa* and *A.niger* (Davis, 2000 and Pel and et al., 2007), have been found to be present in the truffle genome. Among the 74 genes that have been annotated in the *T. melanosporum* genome (Martin et al., 2010), eleven were regulated in the whole genome microarray.

The genes coding for the HSPs were both up-regulated and down-regulated in cold conditions, on the bases of previous data on *S. cerevisiae* (Murata et al., 2006). These functionally conserved proteins prevent protein aggregation and facilitate protein degradation or refolding (Schade et al., 2004). Among those, that were up-regulated, we found Tmelhsp30, Tmelhsp9 and Tmelhspa12a, while Tmelhsp90 and its co-chaperonines were down-regulated. Murata et al. (2006) observed several HSPs (HSP12, HSP104, HSP26, HSP30, SSE2 and SSA4), which were induced in the presence of exposure to 4 °C in *S. cerevisiae*, suggesting a role in the vitalization of enzyme activity. A number of HSPs are also induced when yeast cells are cultured at 4 °C for several months (Homma et al., 2003), suggesting that the induction of various HSPs might be needed to adjust and live at 4 °C. Schade et al. (2004) found HSP genes expressed in late cold response (later than 12 h after exposure to low temperatures). Studies on adaptation to cold in batch cultures of *S. cerevisiae* revealed up-regulation of HSP26 and HSP42 at low temperatures (Schade et al., 2004), while they were down-regulated in chemostat cultures (Tai et al., 2007). Interestingly, the highest fold in qRT-PCR was found for the *Tmelhsp9_1* gene (fold = 104,459), which belongs to the HSP9/12 family.

4.2. Cold treatments elicit a differential expression of genes involved in diverse biological functions

The most up-regulated gene in the array was *TmelDHN1*, which is a single-copy gene, like the homolog in *T. borchii*, while, unlike in other Ascomycetes, dehydrin-homologs are multi-copy genes (Abbà et al., 2006). On the basis of the EST coverage and the expression data of the different life cycle stages (ectomycorrhizae ECM, fruiting body FB, free living mycelium FLM; (Martin et al., 2010), *TmelDHN1* could be a candidate during *T. melanosporum* fruiting body development, as previously seen for *T. borchii*. It has been hypothesized that low temperatures can cause cellular dehydration, and the removal of water from the cytoplasm in the extracellular space, which results in a reduction in the cytosolic volumes and the alteration of cellular mechanisms (Abbà et al., 2006). DHNs are in fact a group of heat-stable plant proteins, which are believed to play a protective role during cellular dehydration (Campbell and Close, 1997), as they have a chaperone-like function which partially stabilizes denatured proteins or membranes, coats them with a cohesive water layer and prevents their coagulation during desiccation (Close, 1997).

We have also observed the induction of cell wall-related genes (Table 2). Among these genes we found a cell wall-related protein, which shows similarity with the *S. cerevisiae* PIR3 protein. Interestingly, this gene resulted to be more expressed in fruiting bodies than in mycelium or ECM tissues such as *TmelDHN1* and *Tmelhsp30_2* (Martin et al., 2010); we suggest that these genes play a role during fruiting body development. In addition, two different putative chitin/polysaccharide

deacetylase genes (*Tmelpda2* and *Tmelpda3*), which could be important in a signalling-regulatory network (Tsigos et al., 2000), are up-regulated. Mention should also be made of the presence of a chitin-binding domain in Tmelpda3 (Balestrini, unpublished). It has been suggested that changes in cell wall proteins during cold stress could be important to maintain cell wall integrity (Yin et al., 2008). However, some members belonging to cell wall-related genes showed a down-regulation (*Tmelscw11, Tmelcrf1, Tmelgut1*) in this whole genome microarray. Transcriptional analysis of *S. cerevisiae* and *Candida albicans* has already shown that the transcriptional level of cell wall protein-encoding genes can vary to a great extent, depending on the cell cycle phase, in which the cell occurs, and on the growth conditions, such as nutrient availability, temperature, pH and hypoxic conditions, which would seem to indicate that cells can adapt the protein composition of the newly formed walls to cope better with the environmental conditions (Yin et al., 2008). A differential expression of cell wall-related genes has also been reported during exposure at 4 °C in *S. cerevisiae* (Murata et al., 2006).

Genes involved in lipid metabolism have been induced in the array experiments. Among them, *Tmelpp02* is involved in the linolenate acid metabolism and its up-regulation could be explained by the changes in the membrane composition. Pedneault et al. (2007) have underlined the effect of temperature on the lipid content and the role of C_{18} on the adaptation to cold temperature stress. Kraus et al. (2004) understood that, in *Cryptococcus neoformans*, membranes were remodelled by fatty acid and sterol metabolism during stress response. The membrane lipids change their desaturation degree, length, configuration and the degree of cyclization of the acyl chains in the presence of temperature stress (Feofilova et al., 2000) in order to maintain membrane fluidity (Steels et al., 1994). Previous works on *T. borchii* demonstrated that the lipid metabolism plays a key role in the reproductive stage (Lacourt et al., 2002 and Gabella et al., 2005); in particular, genes involved in the synthesis of ergosterol have been up-regulated in fruiting bodies (Gabella et al., 2005).

Tmelzrc1 also resulted to be up-regulated in the considered cold condition. On the basis of its function (i.e., a zinc transporter, which is essential for zinc uptake), we suggest a role in homeostasis maintaining.

4.3. Conclusions

Our experiments have demonstrated that the *T. melanosporum* mycelium perceives a temperature change from 25 °C to 4 °C and responds to this environmental change with an important transcription variation.

Genes traditionally listed as environmental stress-responsive genes have been identified as up- and down-regulated in a microarray experiment. Among these, HSPs were the most represented members and their differential expression was validated. Interestingly, other genes, involving dehydration or the lipid metabolism, and already identified among the most expressed genes in the fruiting body of a related truffle (*T. borchii*), have been found to be activated during temperature changes. Similarly to what described for plant cold acclimation (Fowler and Thomashow, 2002), i.e., increase in freezing tolerance in response to low, nonfreezing temperatures, we suggest that truffle must experience a period of cold to initiate the development of truffle ascomata, the formation of which is restricted to winter. Even though fruiting bodies cannot at the present be formed in laboratory conditions, and mycelium still represents the most tractable experimental material, our findings open the way towards novel opportunities to identify the determinants of fungal adaptation to environmental conditions, which could be involved in the interaction with both stimulus-receptors and with downstream proteins, such as HSPs.

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