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Expression and phylogenetic analyses of the Gel/Gas proteins of *Tuber melanosporum* provide insights into the function and evolution of glucan remodeling enzymes in fungi

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ABSTRACT

The β(1,3)-glucanosyltransferases of the GH72 family are redundant enzymes that are essential for the formation and dynamic remodeling of the fungal wall during different stages of the life cycle. Four putative genes encoding glycosylphosphatidylinositol (GPI)-anchored β(1,3)-glucanosyltransferases, designated *TmelGEL1*, *TmelGEL2*, *TmelGEL4* and *TmelGAS4*, have been annotated in the genome of *Tuber melanosporum*, an ectomycorrhizal fungus that also produces a hypogeous fruiting body (FB) of great commercial value (black truffle). This work focuses on the characterization and expression of this multigene family by taking advantage of a laser microdissection (LMD) technology that has been used to separate two distinct compartments in the FB, the hyphae and the asci containing the ascospores. Of the four genes, *TmelGEL1* was the most up-regulated in the FB compared to the free-living mycelium. Inside the FB, the expression of *TmelGEL1* was restricted to the hyphal compartment. A phylogenetic analysis of the Gel/Gas protein family of *T. melanosporum* was also carried out. A total of 237 GH72 proteins from 51 Ascomycotina and 3 Basidiomycota (outgroup) species were analyzed. The resulting tree provides insight into the evolution of the *T. melanosporum* proteins and identifies new GH72 paralogs/subfamilies. Moreover, it represents a starting point to formulate new hypotheses on the significance of the striking GH72 gene redundancy in fungal biology.

1. Introduction

Mycorrhizal fungi are specialized root symbionts that form intimate associations with a great number of plants (Smith and Read, 2008). The most clearly understood function of mycorrhizal symbioses concern the improvement in host mineral nutrient acquisition and the exchange for carbon compounds, which results in positive plant growth responses. Mycorrhizal fungi can also perform several other significant roles, including protection of the plant from biotic and abiotic stresses. In recent years, the availability of the complete genome sequence of fungal species forming mycorrhizae has provided useful information on the organization of their genome and offered the possibility of studying the function of many genes (Martin et al., 2008, 2010).

This work focuses on *Tuber melanosporum* (Ascomycota, Pezizomycotina), an ectomycorrhizal fungus for which the complete genome sequence has recently been published (Martin et al., 2010), giving new insight about the biology of this precious edible fungus (Kues and Martin, 2011). Interest in this fungus in fact not only derives from its symbiotic life, but also from its commercial value since the fruiting body (FB), which is commonly known as black truffle, is an underground mushroom with a particular flavor that makes it a highly prized gastronomic delicacy. Like other mycorrhizal fungi, *T. melanosporum* has a complex life cycle that is characterized by three stages: (i) vegetative growth, during which the fungus undergoes filamentous growth and forms a mycelium, (ii) ectomycorrhizae development, which requires the establishment of a mutual association between the fungus and the host plant and (iii) the formation of the FBs (ascomata) that contain the sexual spores (Murat et al., 2008; Kues and Martin, 2011).

The cell wall is an essential exoskeleton that protects fungi from osmotic shock, determines cell shape and mediates interactions with the environment. In general, the cell wall is composed of a

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network of glucan, mannoprotein and chitin, with variations in levels among different fungal species [for a review see (Latgé, 2007)]. The 125-Mb genome of *T. melanosporum* has shown the presence of many cell wall-related genes (Balestrini et al., 2012; Martin et al., 2010), including 6 genes encoding canonical chitin synthases (*CHSD*, *CHS2*, *CHS3*, *CHS4*, *CHS7* and *CHSG*), 2 genes coding for unconventional myosin-chitin synthases (*CHS6* and *CHS8*), a single gene for both β(1,3)-glucan synthase (*FKSA*) and the regulatory Rho1 GTPase subunit (*RhoA*), as well as several genes encoding β(1,3)-glucan modifying enzymes (Balestrini et al., 2012; Amicucci et al., 2011).

A group of *T. melanosporum* cell wall-related proteins has shown similarity to members of family 72 of glycoside hydrolases (GH) (CAZy; http://www.cazy.org) (Cantarel et al., 2009). To date, GH72 enzymes have only been found in fungi. The importance of these enzymes in fungal biology is related to their role in the remodeling of β(1,3)-glucan, the most abundant polysaccharide in fungal walls and on which both cell wall architecture and mechanical resistance depend.

In general, β(1,3)-glucan is synthesized by a plasma membrane β(1,3)-glucan synthase complex and extruded into the periplasmic space as a linear polymer (Beauvais et al., 2001, 1993; Klis et al., 2006). Glucan chains become branched to form the glucan "core" to which other cell wall components bind by means of extracellular transglycosidases (Latgé, 2007). GH72 enzymes are plasma-membrane GPI-anchored proteins with glucanase/transglycosidase activity. *In vitro*, they internally cleave a laminarioligosaccharide of >10 glucose residues and then transfer the newly generated reducing end to the non-reducing end of another laminarioligosaccharide, forming a new β(1,3)-glycosidic linkage (Mouyna et al., 2000). This transferase activity results in the elongation of linear β(1,3)-glucan chains, or of their branches, thus acting in synergy or alternatively to the β(1,3)-glucan synthase. Experimental evidence indicates that the elongation of branching points of the glucan creates new anchoring sites for other components that are incorporated in the expanding cell wall (Mouyna et al., 2000). Consistent with this, GH72 enzymes preferentially localize in active wall formation sites such as the bud, the septum, the prospore membrane, the tip of the germ tube or the apex of the hypha (Ragni et al., 2007a, 2011; Rolli et al., 2009).

In each fungal species, GH72 enzymes are encoded by a multigene family. The best characterized are the *GEL/GAS/PHR* families from the filamentous fungus *Aspergillus fumigatus* (from *GEL1* to *GEL7*), the budding yeast *Saccharomyces cerevisiae* (from *GAS1* to *GAS5*), the fission yeast *Schizosaccharomyces pombe* (*gas1*$^+$, *gas2*$^+$ *gas4*$^+$ and *gas5*$^+$) and the dimorphic yeast *Candida albicans* (*PHR1*, *PHR2*, *PHR3*, *PGA4* and *PGA5*). The significance of such a redundancy appears to be the requirement of specific isoforms at different stages of the cell cycle or of the life cycle. An example of interplay among various members of a GH72 family during life cycle is provided by the *GAS* multigene family of *S. cerevisiae*. The *GAS1–GAS5* gene pair is expressed during vegetative growth and repressed in sporulation, whereas the *GAS2–GAS4* gene pair shows the reverse pattern (Ragni et al., 2007a, 2007b; Rolli et al., 2009). Consistently with the expression pattern, *Sc*Gas1 and *Sc*Gas5 proteins are required for cell wall formation during vegetative growth, with Gas1 playing the major role while Gas5 only has an auxiliary function. *Sc*Gas2 and *Sc*Gas4 proteins are required for spore wall formation and their combined absence causes severe defects in the sporulation process (Popolo et al., 1993, 1997; Ragni et al., 2007a). *ScGAS3* is weakly expressed during the entire yeast life cycle (Rolli et al., 2010) and encodes an inactive and highly mannosylated protein that is dispensable at any stage of the yeast life cycle (Rolli et al., 2010).

In *A. fumigatus*, the species where β(1,3)-glucanosyltransferases were first identified, *GEL1*, *GEL2* and *GEL4* are expressed during mycelial growth, with *GEL4* being the most expressed of the three genes and also essential for growth (Gastebois et al., 2010).

In *S. pombe gas1*$^+$, *gas2*$^+$ and *gas5*$^+$ expression is cell-cycle dependent (de Medina-Redondo et al., 2008) whereas *gas4*$^+$ expression level is very low during vegetative growth and increases during sporulation. Moreover, *gas1*$^+$ is essential in vegetative growth whereas *gas4*$^+$ is required for ascospore development and viability (de Medina-Redondo et al., 2010) .

Altogether these data underline the important role played by GH72 enzymes in fungal biology (de Medina-Redondo et al., 2008). Although the physiological role of some genes has been described, at least in part, much remains to be established about the role of these enzymes during filamentous fungi development and complex morphogenetic events, such as FB formation. Because of their important role in cell wall biogenesis, GH72 enzymes are also crucial for virulence, in both human and plant fungal pathogens (Calderon et al., 2010; Mouyna et al., 2005; Saporito-Irwin et al., 1995). For example, an involvement of a *GAS1* homolog in plant infections has also been shown in *Fusarium oxysporum*, an ubiquitous soil-borne pathogen (Caracuel et al., 2005). Moreover, *gas1* of *Beauveria bassiana*, an entopathogenic fungus, affects conidial thermotolerance and virulence (Zhang et al., 2011).

Taking advantage of the annotation of four putative GPI-anchored β(1,3)-glucanosyltransferase genes in the *T. melanosporum* genome, we have characterized the GH72 family in this symbiotic Pezizomycetes fungus and their transcriptional profile in the FB and mycelium. In addition, a laser microdissection (LMD) and collection technique was used to separate different tissues in the FB and analyze the expression of GH72 genes in two distinct compartments, the hyphae and the asci. Moreover, by exploiting the rich pool of GH72 proteins currently annotated in the many available fungal genomes, we have investigated in detail the evolutionary history of *T. melanosporum* GH72 proteins in the frame of filamentous fungi diversification.

2. Methods

2.1. Biological materials

The *T. melanosporum* mycelium (Tmel28 strain) was grown in a liquid Modified Melin_Norkrams (MMN) medium in a dark room at 25 °C for 1 month before harvesting. The *T. melanosporum* FBs were purchased from Benvenuto Boasso di Sinio-valle Belbo (CN, Italy), cleaned with a small brush, cut (after removal of the peridium) and then stored at −80 °C.

2.2. RNA Isolation and cDNA synthesis

RNA extraction from the FBs was carried out using an RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions, but replacing the RLT with RLC buffer. Total RNA was extracted from the mycelium using the 'pine tree-method' (Chang et al., 1993). Genomic DNA was removed using a Turbo DNA-free™ reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The cDNA synthesis was described in detail in Balestrini et al. (2012).

2.3. Real-Time quantitative Reverse Transcriptase PCR (Real-Time qRT-PCR)

Gene-specific primers (Table 1) were designed using PerlPrimer software v 1.1.19 (http://perlprimer.sourceforge.net/) and verified by Primer BLAST Software (http://www.ncbi.nlm.nih.gov/tools/primer-last/index.cgi). Prior to Real-Time qRT-PCR, the primers were tested on genomic DNA. Real-Time qRT-PCR was carried out with

Table 1
List of primers used in this study.

Gene	Primer sequences 5′–3′	Application
TmelGEL1	For: ATGAAAGGGCTCACAGCTACTCTCG	Full-length sequence
	Rev: TCAAAAGAGTAGCATGACACCAGC	
	For: GCATTATCTGAACTGTGGGTC	
	Rev: AGAGAAAGAGTGGGATGGAG	Real-Time qRT-PCR, RT-PCR on LMD samples
TmelGEL2	For: ATGAAGCTGCAGCTTTCTGTTCTCGC	
	Rev: CTAAGCCAACCCCCAAAACCCAACA	Full-length sequence
	For: CTAGTAGACACCTTCAAGAACAC	
	Rev: TATACGAGACCACCAGACCA	Real-Time qRT-PCR, RT-PCR on LMD samples
TmelGEL4	For: ATGTGGGCTCGATCGCTCGC	
	Rev: CTATGCAAAAACCAAAGTTGCACC	Full-length sequence
	For: TCTTCGCTGAGTATGGGTG	
	Rev: TCATTTGCTTCCTGGTGGT	Real-Time qRT-PCR, RT-PCR on LMD samples
TmelGAS4	For: ATGCGTTTTACTGTTGCATTTCTTG	
	Rev: TTACAATAAGGCGGCGAACCCAATTG	Full-length sequence
	For: GAACCGACCATTCCTTCAG	
	Rev: GACACCGCTCTTGATATACTC	Real-Time qRT-PCR, RT-PCR on LMD samples
TmelEF1B	For: TCCTACCAGAACGACTTCTC	
	Rev: CCTTCTCAATCGCTCTAACG	Real-Time qRT-PCR, RT-PCR on LMD samples
Tmeltub2	For: GAAATGGAGTTCACTGAGGC	
	Rev: CATCATCAGAGATTCCGGCA	RT-PCR on LMD samples
GAL-GEL1	For: TATAAGCTTAATGAAAGGGCTCACAGCTACTCTC (underlined HinDIII site)	*TmelGEL1* cloning in pYES2
	Rev: CGTCTAGATAGCATGACACCAGCAATCGTTCCC (underlined XbaI site)	

StepOne apparatus (Applied Biosystem). Each PCR reaction was done in a volume of 20 μl containing 1 μl diluted cDNA, 10 μl SYBR Green Reaction Mix and 2 μl of each primer (3 μM stock) using a 48-well plate. The following PCR programme, including the calculation of a Melting curve, was used: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 for 1 min, 95 °C for 15 s. Baseline range and CT values were automatically calculated using the StepOne software. The expression of candidate genes was normalized to that of *TmelEF1B*, used as housekeeping gene, by subtracting the CT value of *TmelEF1B* from the CT value of the candidate gene resulting from the ΔCT. The expression ratio was calculated without the PCR efficiency correction from equation $2^{-\Delta\Delta CT}$, where ΔΔCT represents the $\Delta CT_{sample} - \Delta CT_{control}$. All the reactions were performed for at least two biological and three technical replicates. Statistical analyses were carried out using Rest2008, version 2.0.7, considering data with a p-value < 0.05 as being significantly different.

2.4. cDNA isolation and validation

TmelGEL1, *TmelGEL2*, *TmelGEL4* and *TmelGAS4* cDNA isolation was carried out by PCR amplification from a previously prepared *T. melanosporum* cDNA library (library F2, fruiting body large inserts; Montanini et al., 2011) using the oligonucleotides reported in Table 1. The 50 μl reaction mix contained 10 μl of 5X Phusion HF Buffer, 1 μl of dNTPs (10 mM stock each), 1.5 μl of each primer (10 mM stock), 0.5 μl of Phusion High-Fidelity DNA polymerase (NEB, USA), and 1 μl of diluted plasmid from the library (1:10). PCR amplifications were carried out in a thermal cycler (Biometra) using the following program: 98 °C for 60 s; 35 cycles of 98 °C for 10 s, 57 °C for 10 s and 72 °C for 90 s; 72 °C for 10 min. The amplification reactions were analyzed by agarose gel electrophoresis. DNA fragments were excised from the gel and purified using a GENECLEAN® Turbo Kit (MP Biomedicals, USA), according to the manufacturer's instructions. The purified DNA fragments were cloned in the pGEM-T plasmid (Promega) using T4 DNA Ligase (Promega). The10 μl-reaction contained 50 ng of pGEM-T, 17 ng insert DNA, 1 μl of Ligase 10X Buffer and 1 μl of T4 DNA Ligase (10–20 U/μl). The plasmids were transformed into One-Shot electrocompetent TOP10 *E. coli* cells (Invitrogen) using a MicroPulser™ electroporator (BioRad). The plasmids were purified from positive colonies using a QIAprep Spin Miniprep Kit (QIAGEN) and then sent to the Sequencing service at Munich University (Database ID: http://www.gi.bio.lmu.de/sequencing). The amino acid sequences deduced from the nucleotide sequences were analyzed using different software.

2.5. Laser microdissection, RNA extraction and RT-PCR

FB pieces (gleba) were fixed in freshly prepared Farmer's [Ethanol:Acetic Acid (3:1)] fixative at 4 °C overnight for paraffin embedding. A Leica AS Laser Microdissection system (Leica Microsystems, Bensheim, Germany) was used to isolate different tissues from the prepared sections following the described previously cut conditions (Balestrini et al., 2007). About 1000 asci (containing ascospores) and about 22 pieces of the hyphal tissue (300 μm × 150 μm) were collected for each cell-type population in a single tube. The amount for each biological replicate from one treatment was collected in 1 day. After addition of 50 μL of RNA extraction buffer from the PicoPure kit (Arcturus Engineering, Mountain View, CA, USA), samples were incubated at 42 °C for 30 min, centrifuged at 800*g* for 2 min and then stored at −80 °C. Total RNA extractions were performed adopting a slightly modified PicoPure kit protocol (Arcturus Engineering) as described by Balestrini et al. (2007). A One-Step RT-PCR kit (Qiagen, Valencia, CA, USA) was used for the Reverse Transcriptase PCR (RT-PCR) experiments, which were conducted on the RNA extracted from several samples. Reactions were carried out as described in detail in Balestrini et al. (2007) using the oligonucleotides reported in Table 1. The RT-PCR experiments were conducted on at least three independent biological replicas and two technical replicates.

2.6. Phylogenetic analyses

We analyzed the GH72 proteins of the three major groups of Ascomycota (Saccharomycotina, Pezizomycotina and Taphrinomycotina) as well as of some Basidiomycota fungi, used as outgroup species (Martin et al., 2010; Medina et al., 2011). The GH72 pro-

teins of Saccharomycotina were selected from the CAZy database (April, 2012) (http://afmb.cnrs-mrs.fr/CAZY/index.html) (Coutinho and Henrissat, 1999) or using BlastP against the non-redundant "nr" protein database available at NCBI (April, 2012) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). On the contrary, the genomic assemblies and the related Genome Databases were queried to identify the entire complement of GH72 proteins of two Taphrinomycotina (*Schizosaccharomyces japonicus* and *Schizosaccharomyces pombe*), five Basidiomycota (*Cryptococcus gattii*, *Cryptococcus neoformans*, *Melampsora larici-populina*, *Puccinia graminis*, *Ustilago maydis*) and 23 Pezizomycotina belonging to all five classes of Sordariomycetes, Leotiomycetes, Eurotiomycetes, Dothideomycetes and Pezizomycetes. Supplemental Table S1 reports the list of all the analyzed GH72 sequences, together with sequence abbreviations, species classification and information on the protein domain structure, such as the presence/absence of the Cys-box and the C-terminal low complexity regions. The list of all queried Genome Databases is reported in Supplemental Table S2.

A preliminary alignment of the 252 initially selected GH72 proteins, belonging to 56 species, revealed the presence of 13 partial or anomalous sequences lacking highly conserved protein regions or containing extra portions from unrelated proteins. These 13 sequences were excluded from further analyses since most of them are "hypothetical" proteins not yet experimentally validated, likely derived from incorrect gene annotation.

The final dataset includes 239 proteins belonging to 24 Pezizomycotina, 25 Saccharomycotina, 5 Basidiomycota and two *Schizosaccharomyces* (Taphrinomycotina). For each analyzed species, Supplemental Table S2 reports the number of GH72$^+$ and GH72$^-$ proteins, characterized by the presence and absence of the Cys-box, respectively, together with the species classification. The 114 GH72$^+$ and 125 GH72$^-$ protein sequences were aligned separately with MUSCLE (Edgar, 2004) and each alignment was manually optimized. The two multi-alignments were then merged with the "alignment profile" option of Geneious (Drummond et al., 2011). The total alignment of the all GH72 proteins, hereafter called "total_GH72", was again manually improved, and the two GH72$^+$ *Cryptococcus* (Basidiomycota) proteins were removed because of their high sequence divergence from the other species. Therefore, the final number of analyzed sequences was 237.

Evolutionary analyses were carried out on the most reliable portion of each alignment, thus excluding all the gap-containing regions as well as the highly variable N-terminal and C-terminal protein regions. Therefore, a total of 379, 287 and 266 amino acid sites were analyzed in the GH72$^+$, GH72$^-$ and the "total_GH72" alignments, respectively. ProtTest 3 was used to select the evolutionary model best-fitting to each alignment, choosing among 64 candidate models and on the basis to the AIC and BIC selection criteria (Abascal et al., 2005). The LG model, plus a gamma distribution for rate heterogeneity across sites (+G), was selected for the "total_GH72" alignments, while WAG + G and WAG + G + F (*F* = observed amino acid frequencies) models were selected for the GH72- and the GH72$^+$ alignments, respectively. Phylogenetic reconstructions were performed according to the Maximum Likelihood (ML) method using phyML 3.0 (Guindon et al., 2010), with bootstrap values based on 100 replicates. The Basidiomycota sequences were used as outgroup taxa in the analysis of the total_GH72 alignment.

Low complexity (LC) protein regions were identified using the SEG program, with default parameters (w = 12; k1 = 2.2; k2 = 2.5) (Wootton and Federhen, 1993) (http://mendel.imp.ac.at/METHODS/seg.server.html). The Ser/Thr-rich domains were defined as the LC regions present at the C-terminal portion of the protein and having a high Ser/Thr content. The presence and location of signal peptide cleavage sites was predicted using SignalP 4.0 (Petersen et al., 2011) (Database ID: http://www.cbs.dtu.dk/services/SignalP/). The potential GPI lipid modification sites were predicted using the "big-Pi fungal predictor" (Database ID: http://mendel.imp.ac.at/gpi/fungi_server.html) (Eisenhaber et al., 2004).

2.7. Construction of an S. cerevisiae strain for the heterologous expression of T. melanosporum GEL1

The *TmelGEL1* complete coding sequence was PCR-amplified from the full-length *TmelGEL1* cDNA and cloned into the pGEM-T vector using the GAL-GEL1 pair of primers (Table 1). The fragment of about 1.35 kbp was double digested with *Hin*dIII and *Xba*I and cloned in the similarly digested pYES2 vector to obtain pYES-TmelGEL1 harboring the *GEL1* coding sequence downstream the inducible yeast *GAL* promoter. The absence of errors and the correct insertion were confirmed by DNA sequencing. The W303-1B (*MATα*, *ade2-1 his3-11*, *15 trp1-1 ura3-1 leu2-3*, *112 can 1-100*) and the derived WB2d (*gas1::LEU2*) yeast strains were transformed with pYES2 or pYES-*TmelGEL1* (Gietz et al., 1995). Cells were grown at 30 °C in YNB-glucose minimal medium (SD: 6.7 g of Difco yeast nitrogen base [without amino acids] per liter and 2% of glucose supplemented with 50 mg/of the appropriate amino acids, 100 mg of adenine per liter and 2% of agar for the solid medium). The transformants were grown in liquid SD and then shifted for 48 h to fresh minimal medium containing 2% galactose (SGal). The sensitivity to Calcofluor White, a cell wall perturbing agent, was tested as described previously (Ragni et al., 2007b).

RT-PCR was used to monitor the presence of the *TmelGEL1* transcript. Total yeast RNA was extracted using RNeasy Plant Mini Kit (QIAGEN) and treated with TurboDNase (AMBION, standard protocol). The primers used are listed in Table 1. The 20 μl reaction mix contained: 4 μl of 5X buffer, 1 μl dNTPs (stock: 10 mM each), 0.5 μl of each primer (1 μg/μl), 0.5 μl of One-Step RT-PCR enzyme mix (QIAGEN), and 1 μl of RNA (∼ 30 ng). RT-PCR reactions were carried out in a thermal cycler (Biometra), using the following program: 50 °C for 30 min; 95 °C for 15 min; 35 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; 72 °C for 7 min.

3. Results

3.1. Organization of the multigene family encoding GH72 enzymes in T. melanosporum

Four paralogous genes encoding β(1,3)-glucanosyltransferases of the GH72 family were annotated in the genome assembly of *T. melanosporum* (hereafter abbreviated as *Tmel*) (http://mycor.nancy.inra.fr/IMGC/TuberGenome/) (Balestrini et al., 2012). These genes were designated *TmelGEL1*, *TmelGEL2*, *TmelGEL4* and *TmelGAS4* according to the best characterized and most closely related proteins identified by phylogenetic reconstructions (see below). In this work, we have experimentally validated the whole protein-coding sequence of these genes by means of cDNA isolation and sequencing, whereas the 5′- and 3′-UTRs were not determined.

The four *T. melanosporum* GH72 genes are located on different genomic scaffolds. The analysis of the gene structure, which was limited to the coding-portion, revealed a different intron–exon organization for the four paralogs. The gene organization scheme, reported in Supplemental Fig. S1, shows that the exon number ranges from 4 (in *TmelGAS4*) to 7 (in *TmelGEL1*). The exon size is very heterogeneous (from the 12 bp of exon 1 of *GEL2* to the 1096 bp of exon 5 of *GEL2*), while the intron length is quite uniform (average 60 ± 9 bp; range 47–76 bp). The introns represent from 11 (*TmelGAS4*) to 21% (*TmelGEL1*) of the whole gene length and all follow the "GT-AG" rule.

3.2. Features of the Gel/Gas family of proteins of T. melanosporum

As shown in Fig. 1A, all Gel/Gas proteins of *T. melanosporum* share a typical GH72 modular structure that is composed of: (i) an N-terminal signal sequence ranging from 18 to 22 amino acids; (ii) a GH72 catalytic domain (Pfam family: PF03198); (iii) a "linker" region (L) characterized by the presence of a conserved Cys that is usually connected to the central Cys of the GH72 domain (Popolo et al., 2008; Hurtado-Guerrero et al., 2009); (iv) a C-terminal low complexity (LC) region ranging from 19 to 34 amino acids and with a Ser/Thr percentage of 16–53% and (v) a C-terminal signal for GPI attachment, ranging from 26 to 34 amino acids. Moreover, TmelGel4 has an additional cysteine-rich domain, named the Cys-box, also known as carbohydrate-binding module of family 43 (CMB43 in the CAZy database) or X8 domain (Pfam: PF07983). This module is unique to fungal and plant proteins. In fungi it is characterized by the presence of a pattern of 8 conserved Cys residues (8Cys-box) connected by 4 intra-molecular disulfide bonds, as shown in Fig. 1A for Gel4 (Popolo et al., 2008). According to whether the Cys-box is present or absent and based on a previous GH72 family classification (Ragni et al., 2007), TmelGel4 belongs to the $GH72^{+}$ subfamily, while, TmelGel1, TmelGel2 and TmelGas4 belong to the $GH72^{-}$ subfamily. In the fungal $GH72^{+}$ enzymes, the GH72 domain and the Cys-box physically interact and are both essential for proper folding and activity (Hurtado-Guerrero et al., 2009; Popolo et al., 2008). In plants, the Cys-Box is shorter and contains only 6 Cys residues engaged in the formation of 3 intra-domain disulfide bonds (6Cys-box) (Barral et al., 2004, 2005). Moreover, it is an independent β(1,3)-glucan binding domain appended to a catalytic module (Palomares et al., 2003).

The multi-alignment of the *Tmel* proteins with 237 selected homologs of Ascomycota and Basidiomycota led to the identification of some conserved protein features. As expected, the catalytic GH72 domain of all the *Tmel* proteins contains two conserved Glu residues and three Tyr residues that define the active site (Fig. 1A). Moreover, the five conserved Cys residues of the GH72 domain, together with the conserved Cys of the linker, are predicted to form a cluster of three intra-molecular disulfide bonds as in other GH72 enzymes (Fig. 1A) (Popolo et al., 2008; Hurtado-Guerrero et al., 2009). The two conserved Glu residues are essential for catalysis and are located in a bipartite motif separated by about 100 amino acids. The frequency plot of this motif, calculated on all of the analyzed 237 proteins using WebLogo (Crooks et al., 2004) shows the presence of some invariable amino acids in addition to the two essential Glu residues (Fig. 1B). Moreover, the bipartite motif does not show significant differences between the $GH72^{+}$ and $GH72^{-}$ subfamilies.

Almost all the analyzed GH72 proteins have a C-terminal low complexity (LC) region, just before or including the GPI attachment site (also named ω site). Interestingly, the C-terminal LC region of *T. melanosporum* and other Pezizomycotina is highly variable both in length and Ser/Thr content, and does not contain long Ser/Thr stretches. On the contrary, in the *Schizosaccharomyces* genus and in almost all Saccharomycotina proteins this LC region is very long and rich in Ser/Thr stretches (minimum repeated unit size: 5 amino acids), and consequently it has been named "poly Ser/Thr region". In 75 out of the 109 analyzed Saccharomycotina proteins, the LC region shows an average length of 43 ± 14 amino acids and a Ser/Thr percentage of 61 ± 11%, and therefore it constitutes a "poly Ser/Thr region" (see the dashed blocks in Fig. 2).

3.3. Phylogenetic analyses

The phylogenetic tree of Fig. 2 describes the evolutionary relationships between the four *T. melanosporum* GH72 proteins and the homologous sequences of 50 other Ascomycota fungi, using as outgroups 3 species of Basidiomycota. This tree contains 237 sequences and includes the full complement of GH72 proteins identified in the genome assemblies of 23 Pezizomycotina, 2 Taphrinomycotina (*Schizosaccharomyces*) and 3 Basidiomycota species (see Section 2 and Supplemental Tables S1 and S2). Compared to the phylogenetic tree of only 70 GH72 proteins reported in Ragni et al. (2007), our analyses comprise 38 previously un-sampled fungi, including *T. melanosporum* which is here the only species representative of Pezizomycetes, the earliest diverging lineage within the Pezizomycotina (Martin et al., 2010).

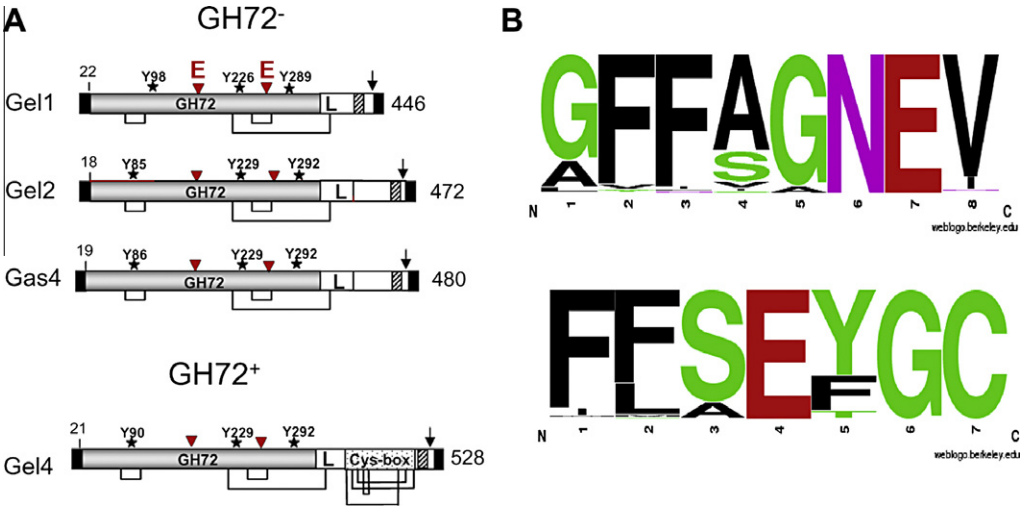

Fig. 1. Features of the *T. melanosporum* GH72 family and of the bipartite catalytic motif. (A) The GH72 domain, the connecting linker (L), the LC region (stripped box) and the Cys-Box (dotted box) are indicated. The potential catalytic glutamic acid residues (E) are represented in red by a triangle. These residues are located in the following conserved bipartite motifs where they are indicated in bold: in Gel1 (151-FFAAN**E**VI- and 254-FLS**E**FGC-), in Gel2 (153-FFAGN**E**VV and 257-FFS**E**FGC), in Gas4 (154-FLAGN**E**VIF and 257-FFT**E**YG) and Gel4 (154-FAGN**E**VSN and 257-FFA**E**YGC). The tyrosines of the active site are marked by a star. The arrow marks the predicted GPI-attachment site (in Gel1: G414; in Gel2: G441; in Gas4: N455; in Gel4: N495). The limits of the LC region are in Gel1: 394–431, in Gel2: 425–443, in Gas4: 419–455 and in Gel4: 419–452. The connection, by means of a disulfide bond, between two Cysteine residues is indicated with a line in the lower part of each scheme. The position of the S-S bridges is inferred from the experimentally determined map of disulfide bonds of the *S. cerevisiae* Gas1 and Gas2 proteins (Popolo et al., 2008). (B) The logo of the bipartite catalytic motif, generated with WebLogo (http://weblogo.berkeley.edu/logo.cgi), shows the residue frequencies calculated on 237 Ascomycota and Basidiomycota proteins (see Table S1). The catalytic glutamate residues are shown in red; hydrophobic residues in black; hydrophilic residues in green; and asparagine residues in purple-violet.

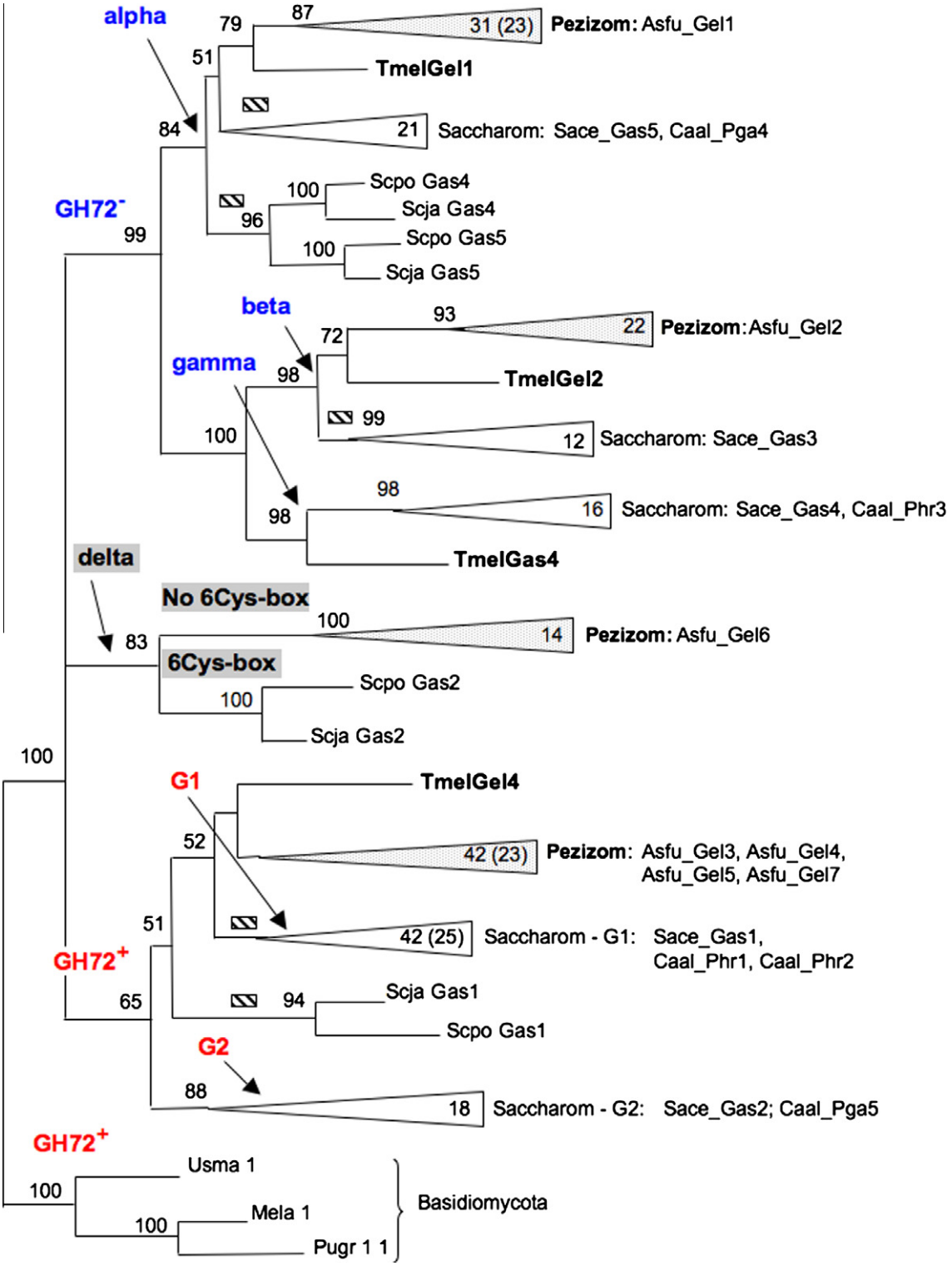

Fig. 2. ML evolutionary tree of the GH72 proteins of Ascomycota. Large clades were reduced to a triangular branch, with the number inside the triangle corresponding to the number of sequences, and the number in brackets corresponding to the number of species (only when the sequence number is different from the species number). The best-characterized proteins of each clade are also listed. The dashed blocks indicate the presence of a C-terminal poly Ser/Thr region (see text). Red and blue colors indicate paralogous genes of the GH72$^-$ and GH72$^+$ subfamilies, respectively, while gray background indicates the new delta subfamily. Only bootstrap values > 50% are shown. The unreliable basal node was collapsed to a tritomy. Basidiomycota species were used as outgroup taxa. Branch length is proportional to the number of substitutions per site. Pezizom: Pezizomycotina; Saccharom: Saccharomycotina. Species abbreviations: Asfu: *Aspergillus fumigatus*; Caal: *Candida albicans*; Mela: *Melampsora larici-populina*; Pugr: *Puccinia graminis*; Sace: *Saccharomyces cerevisiae*; Scja: *Schizosaccharomyces japonicus*; Scpo: *Schizosaccharomyces pombe*; Tmel: *Tuber melanosporum*; Usma: *Ustilago maydis*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Each *Tmel* proteins lacking the Cys-box (TmelGel1, TmelGel2 and TmelGas4) is located in a distinct clade of the GH72$^-$ subfamily. These clades are highly supported (bootstrap values $\geqslant$ 84% in Fig. 2) and have been named alpha, beta and gamma (Fig. 2). In both the alpha and beta clades, the phylogenetic relationships expected between the main Ascomycota groups are fulfilled. Indeed,

according to the fungal phylogeny, *T. melanosporum* (Pezizomycetes, Pezizales) is the basal branch of Pezizomycotina, with Saccharomycotina sister taxon of Pezizomycotina, and *Schizosaccharomyces* (if present) basal to all other Ascomycota (Martin et al., 2010; Medina et al., 2011). Moreover, *Schizosaccharomyces* species show the duplication of the alpha GH72^{-} protein, and the loss of the beta GH72^{-} paralog (Fig. 2). Surprisingly, the gamma clade comprises only Saccharomycotina species and *Tmel*Gas4 as the sole Pezizomycotina representative. Since the evolutionary tree in Fig. 2 includes the full GH72 complement of 24 Pezizomycotina and 2 *Schizosaccharomycetes* species, the absence of gamma GH72^{-} in the above-mentioned taxa cannot be considered an artefact caused by the accidental loss of analyzed sequences. Therefore, the most parsimonious hypothesis is that, the gamma GH72^{-} protein was prematurely lost in the ancestor of the main Pezizomycotina lineages, except for Pezizales. Finally, the beta and gamma clades are sister groups and both lack *Schizosaccharomyces* representatives (Fig. 2). Therefore, we can hypothesize that the alpha and gamma paralogs have arisen from a gene duplication event occurred in the common ancestor of Saccharomycotina and Pezizomycotina, but after the divergence from Taphrinomycotina.

TmelGel4, a Cys-box containing protein, groups within the GH72^{+} subfamily and forms the most basal branch of all other Pezizomycotina species (bootstrap < 50% in Fig. 2). Interestingly, the GH72^{+} subfamily has only a moderate support (65% bootstrap in Fig. 2) and consists of: (a) one Pezizomycotina clade (including TmelGel4); (b) two distinct Saccharomycotina clades, which have been named G1 and G2, since they comprise *S. cerevisiae* Gas1 and Gas2, respectively. Unfortunately, the evolutionary history of the GH72^{+} subfamily cannot be fully reconstructed because most of the internal nodes are unresolved (bootstrap values $\leqslant$ 50%) and even the basal node is moderately supported (65% bootstrap). However, two plausible evolutionary scenarios can be envisaged for the existence of the G1 and G2 Saccharomycotina clades: (a) a GH72^{+} gene duplication occurred only in the ancestor of Saccharomycotina; or (b) a GH72^{+} gene duplication occurred in the common ancestor of Saccharomycotina and Pezizomycotina, followed by gene loss in Pezizomycotina.

Together with the GH72^{+} and GH72^{-} subfamilies, our evolutionary analysis has identified a third highly supported clade, named "delta" (83% bootstrap in Fig. 2). This clade consists of the Gas2 proteins of two *Schizosaccharomyces* species (one of which was indicated as Scpo_3 in Ragni et al., 2007) and of a large group containing representatives of four out of the five major Pezizomycotina classes. Thus, the delta protein is absent in the fifth Pezizomycetes lineage to which the *Tuber melanoporum* belongs (Fig. 2 and Supplemental Fig. S3). The delta proteins have some unusual features. In particular, the *Schizosaccharomyces* Gas2 proteins show: (1) a modified Cys-box domain (named 6Cys-box), which lacks the 2nd and 8th conserved Cys, and the predicted interconnecting disulfide bond; (2) the lack of the C-terminal poly Ser/Thr region; and (3) the lack of the C-terminal signal for GPI attachment. Thus, the *Schizosaccharomyces* Gas2 proteins can be considered as "truncated" GH72^{+} proteins with a Cys-box more similar to the plant Cys-box (Barral et al., 2004, 2005) (see Section 4). The "delta" GH72 proteins of Pezizomycotina have neither a standard Cys-box (8Cys-box) nor a modified 6Cys-box, and show a modified bipartite motif surrounding the two conserved catalytic Glu residues (Fig. 1B). Indeed, among all GH72 proteins only the delta Pezizomycotina have the (I/V)GNE and the SETG sequences around the catalytic Glu. Thus, there is a conservative A → I/V substitution in the first part of the bipartite motif (Fig. 1B), and a significant non-conservative Y/F → T substitution (loss of an aromatic residue) in the second part of the bipartite motif (Fig. 1B). Therefore, it could be very interesting to investigate the specific function of the delta GH72 proteins of Pezizomycotina (for example, *A. fumigatus* Gel6 of Fig. 2). It should also be noted that the basal polytomy of our phylogenetic tree and the fairly small resolution of the GH72^{+} cluster leaves the possibility that the delta clade and the GH72^{+} subfamilies are sister groups, thus making the delta paralog a transition form from the GH72^{+} to the GH72^{-} subfamily.

Finally, it is worth noting that only one Cys-box-containing GH72 protein (i.e., GH72^{+} protein) and no GH72^{-} proteins have been found in all analyzed genomes of Basidiomycota (Fig. 2), here representing the outgroup. On the contrary, *T. melanosporum* and other Ascomycota have both GH72^{+} and GH72^{-} proteins. Thus, we suggest that GH72^{+} could be the ancestral GH72 gene, and that it gave rise to the GH72^{-} subfamily of Ascomycota through gene duplication and loss of the Cys-box domain.

As detailed in Supplemental Figs. S2 and S3, further gene duplications have recently occurred in some ascomycetes lineages. Indeed, in the GH72^{+} subfamily, a gene duplication can be hypothesized in the G1 Saccharomycotina clade, since this group includes two GH72^{+} proteins for 18 of the 25 represented species (Supplemental Fig. S2; see also differences between the species and sequence number in Fig. 2). Moreover, up to two gene duplications occurred in GH72^{+} of Pezizomycotina (Eurotiales), giving rise, among others, to the four proteins of *A. fumigatus* (*Asfu* Gel3, Gel4, Gel5, and Gel7). These duplications certainly occurred after the separation of Eurotiales from Onygenales, as there is a single GH72^{+} protein in the genomic sequence of the three analyzed Onygenales fungi (Supplemental Fig. S2; note the differences between the species and sequence number in Fig. 2). With regard to the GH72^{-} subfamily, in the alpha clade there are two different GH72 proteins in each analyzed species of the three closely related classes of Leotiomycetes, Dothideomycetes and Sordariomycetes (except for *Fusarium/Gibberella*) (Supplemental Fig. S3). Thus, in spite of the low resolution of the internal nodes of the alpha clade, these data suggest a duplication event in the common ancestor of the three above-mentioned classes, followed by gene loss in some Sordariomycetes species (Supplemental Fig. S2).

Overall, our evolutionary analysis shows that the GH72^{+} and the GH72^{-} subfamilies have undergone both multiple duplications and well-delimited losses of genes, although at very different times. Indeed, in GH72^{-} there is a prevalence of ancient duplications that occurred in the early phases of Ascomycota evolution (giving rise to the alpha, beta and gamma paralogs), while a more recent duplication is observed only in the alpha clade, i.e., in the ancestor of three Pezizomycotina classes. On the contrary, in GH72^{+} the most ancient duplication event originated the G1 and G2 paralogs of Saccharomycotina (this event cannot be exactly mapped because of the limited tree resolution), but three more recent duplications occurred in Saccharomycotina (G1 group) and in Pezizomycotina Eurotiales.

3.4. Transcriptional profiling of the β(1–3)glucanosyltransferases-encoding genes in T. melanosporum

It was previously observed that members of the β(1,3)glucanosyltransferase family are not strongly regulated during ectomycorrhizae development (Balestrini et al., 2012). In this work we examined the FB, a different developmental stage. As shown in Table 2, gene expression experiments were performed to evaluate the expression level of GH72 genes in the FB *versus* the free-living mycelium (FLM). Both microarray and Solexa data, originated in the frame of the *T. melanosporum* genome project (Martin et al., 2010), point to a common trend in the expression levels of the genes. Out of the four genes, *TmelGEL1* has the least abundant transcript both in FLM and in FB (Table 2). However, by comparing FB to FLM, *TmelGEL1* is strongly up-regulated while *TmelGEL4* and *TmelGAS4* seem to be down-regulated and *TmelGEL2* is only slightly up-regulated. In this study we have validated the above-men-

tioned data by Real-Time qRT-PCR on the FB, and provided results that are consistent with the transcriptomics data. *TmelGEL1* and *TmelGEL2* transcript levels are significantly increased whereas *TmelGEL4* and *TmelGAS4* transcript levels are unchanged and slightly decreased, respectively (Table 2, *last column*). This could result from regulation of transcription and/or mRNA stability/degradation.

Truffle FBs, named ascomata, are composed of different cytological components that are expected to be functionally different: an outer peridium and an inner gleba, which consists of vegetative hyphal cells and reproductive structures (asci and ascospores). In order to determine the expression profiles for the four GH72 genes in different compartments, we applied the laser microdissection (LMD) technology to separate the reproductive structures (asci containing ascospores) from the vegetative hyphae forming the gleba (Fig. 3A–C).

In the RT-PCR experiments on the microdissected samples, an amplified fragment of the expected size was observed in both the cell types tested using specific primers for the transcript of the housekeeping gene *TmeleEF1B* (XP_002838562.1), encoding the Elongation Factor 1B (Fig. 3D). The absence of an amplified product in the RT-minus reactions excluded genomic DNA contamination (data not shown). An additional control was conducted using specific primers for a β-tubulin gene (*TmelTub2*; XP_002840160.1) and again a specific band was present in the two LMD samples (Fig. 3D). On the other hand, the *TmelGEL1* transcript was detected only in the vegetative region and not in the reproductive structures (Fig. 3D). Moreover, the amplified band was faint suggesting that, despite this gene is up-regulated in FB, its transcript level is low as also indicated by the transcriptomics data (Table 2). To rule out that the total RNA used was too low, a double amount of RNA from the reproductive structures was also tested, but again *TmelGEL1* transcript was not detected in this compartment. This result indicates that the *TmelGEL1* is either not expressed or expressed at a not detectable level in the reproductive structures. About the other three genes, the corresponding transcripts were detected in both the compartments (Fig. 3D). These results, combined with the expression levels reported in Table 2, indicate that *TmelGEL4*, *TmelGAS4* and *TmelGEL2* genes are expressed in FLM and FB tissues. By contrast, *TmelGEL1*, showed a different expression pattern with a weak expression in FLM and strong induction in FB, where the transcripts have been located uniquely in the hyphal compartment. Thus, a specific role could be suggested for *TmelGEL1* with respect to the other GH72 genes (see Section 4).

3.5. Test of the capability of TmelGEL1 to complement the *gas1Δ* mutation of *S. cerevisiae*

To perform functional experiments in yeast, we have focused our attention on *TmelGEL1* since it is the most up-regulated GH72 gene in the truffle FB and is specific of the vegetative hyphae compartment. In addition, *TmelGEL1* clusters in the alpha clade (Fig. 2) together with *A. fumigatus GEL1* that is able to fully complement *S. cerevisiae gas1Δ* phenotype (Mouyna et al., 2005). The *gas1Δ* mutant was transformed with the pYES2 vector harboring the *TmelGEL1* coding sequence under the control of the *GAL* promoter. Cells were examined in repressing (glucose) or inducing conditions (galactose). We used RT-PCR to test the presence of the *TmelGEL1* transcript in the yeast cells. Whereas no amplification product was found in the RNA extracted from the repressing condition, a specific band was detected in the retrotranscribed total RNA extracted from the induced cells, indicating that *TmelGEL1* was expressed (data not shown). However, none of the typical phenotypic traits of the mutant strain was restored to normality after induction. The examined phenotypic defects included: the cell morphology (large and round in the mutant), growth rate (lower in the mutant) and Calcofluor sensitivity (high in the mutant). Thus *Tmel*Gel1 protein, if produced and matured, is not able to complement the *gas1Δ* mutation in yeast.

4. Discussion

In this work, we validated and characterized the multigene family encoding β(1,3)glucanosyltransferases (GH72 enzymes) in the symbiotic and edible fungus *T. melanosporum*. The family comprehends four intron-containing genes, *GEL1*, *GEL2*, *GEL4* and *GAS4*. Consistently with the structures of *T. melanosporum* and other fungal genes, but unlike from higher eukaryotes, these introns are quite small (Martin et al., 2010). On the contrary, the exons are longer and of heterogeneous size. It is interesting noting that, despite the contraction of the size of several gene families observed in *T. melanosporum* genome (Martin et al., 2010), four *TmelGEL/GAS* genes were retained. This supports the notion that GH72 redundancy, typical of this enzyme family, is required for the basic and vital functions of this fungus.

TmelGel1, *Gel2* and *Gas4* belong to the $GH72^{-}$ subfamily, whereas *TmelGas4* is a member of the $GH72^{+}$ subfamily. In the TmelGel4 protein a cluster of three disulfide bonds is predicted in the GH72 domain plus linker region. The additional 8 Cys residues, present in the Cys-box, are likely engaged in the formation of a second cluster of four disulfide bonds, similarly to the *S. cerevisiae* Gas1 protein as depicted in Fig. 4 (Popolo et al., 2008; Hurtado-Guerrero et al., 2009). All four *T. melanosporum* paralogous proteins contain potential N-linked glycosylation sites and a high percentage of serine and threonine residues that could be the target of O-mannosylation, indicating that these proteins are likely to be glycosylated. In addition they are predicted to be GPI-anchored.

The evolutionary analysis here described has shown that GH72 constitute an ancient and wide family, whose genes have undergone several steps of gene duplication and/or loss and have often given rise to taxon-specific proteins (Fig. 2). This analysis has been performed on *T. melanosporum*, as the only available species of Pezizomycetes (the basal Pezizomycotina lineage), other 50 filamentous fungi, almost widely distributed among the main Ascomycota lineages, and three Basidiomycota species used as outgroups. Thus, our evolutionary analyses concern a very large taxon sample with a good representativeness of the fungal diversity,

Table 2
Gene expression data. Microarray and Solexa data are in accordance to Martin et al. (2010). The expression of candidate genes in qRT-PCR experiments was normalized to that of *TmelEF1B*, used as housekeeping gene.

Name	Microarray			Solexa (RPKM)		Fold-change qRT-PCR (±SD)
	FB	FLM	Ratio FB/FLM	FB	FLM	
TmelGEL1	2004.581	9183	218.3	8.5	0.8	12.83 ± 2.64
TmelGEL2	8549.231	5814.170	1.5	88.6	48.9	3.84 ± 1.37
TmelGEL4	8990.506	18260.602	0.5	62.1	237.2	1.04 ± 0.04
TmelGAS4	7684.884	14993.559	0.5	35.3	338.4	0.34 ± 0.41

RPMK: reads per kb per million reads. FB: fruiting body. FLM: free-living mycelium.

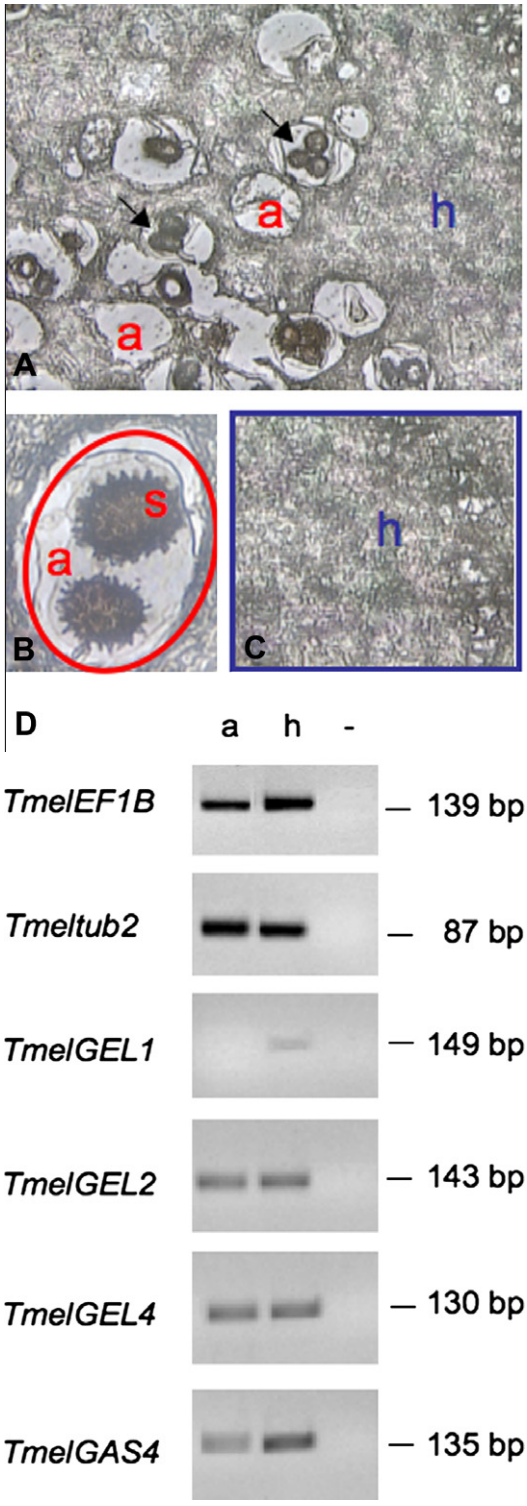

Fig. 3. Laser microdissection on the *T. melanosporum* fruiting body. (A) Section of the inner part (gleba) of a *T. melanosporum* fruiting body. The vegetative hyphae (h) and reproductive structure (asci, a) can be distinguished. (B and C) Magnification of the compartments collected separately using the LMD: ascus containing the ascospores (B) and hyphal region (C). In B the circle marks the tissue collected using the LMD. a, asci; h, hyphae; s, ascospores. (D) RT-PCR analysis of the microdissected samples (asci containing the ascospores, a; hyphae, h; -, negative control). *TmelEF1B* and *Tmeltub2* primers amplified DNA fragments of the expected size in both the samples. Several PCR cycles have been used to verify the efficiency of PCR and the RNA amount in the two LMD samples. Using specific primers for *TmelGEL1*, *TmelGEL2*, *TmelGEL4* and *TmelGAS4*, a fragment of the expected size can be observed in both LMD samples, except for *TmelGEL1* where the corresponding transcripts can only be detected in the hyphal compartment.

greatly extending previous GH72 phylogenetic studies carried out by Ragni et al. (2007).

The two main subfamilies GH72^{+} (with a Cys-box) and GH72^{-} (without a Cys-box), identified by Ragni et al. (2007), have been here confirmed and more deeply characterized, allowing the identification of of numerous paralogous proteins within each subfamily (for example, the alpha, beta and gamma GH72^{-} proteins; G1 and G2 Saccharomycotina-specific GH72^{+} proteins; and so on). Remarkably, our analysis does not support the existence, proposed by Gastebois et al. (2010) on the basis of a small GH72 protein number, of two distinct GH72^{+} groups characterized by the presence/absence of a C-terminal Ser/Thr-rich region. On the contrary, we have found that a C-terminal Low Complexity (LC) region rich in Ser/Thr is present only in Saccharomycotina and in some *Schizosaccharomycetes* proteins (see dashed blocks in Fig. 2), while in Pezizomycotina the C-terminal LC region shows a highly variable Ser/Thr content.

As important novelty, beyond GH72^{+} and GH72^{-} subfamilies, we have here identified a new taxon-specific GH72 subfamily, named delta. This new group includes only sequences of some fungi, i.e., the Gas2 proteins of the *Schizosaccharomyces* genus, containing a modified 6Cys-box rather than the standard 8Cys-box, and the Pezizomycotina proteins lacking any type of Cys-box (neither the 8Cys-box nor the 6Cys-box). Notably, in Pezizomycotina these delta proteins are present in many species of four out the five main lineages, except in *T. melanosporum*, which is the only available Pezizomycetes species. This suggests relevant differences in cell wall remodeling and biogenesis between *Tuber* and the remaining Pezizomycotina fungi. Fig. 4 shows that the Cys-box disulfide bonds network predicted for the delta *S. pombe* Gas2 protein is very similar to the mapped network of the Cys-box from Ole e 9 protein of olive tree (*Olea europaea*) pollen, the best known member of the GH17 family of long β(1,3)-glucanases (Popolo et al., 2008). Thus, the 6Cys-box of the GH72 delta subfamily constitutes an interesting exception. Indeed, in plant this domain functions as a non-catalytic β(1,3)-glucan binding module (Palomares et al., 2003; Barral et al., 2005) that binds the β(1,3)-glucan of callose during pollen growth (Barral et al., 2005). Interestingly, the *S. pombe* Gas2 protein, which also lacks GPI, is expressed during vegetative growth and remains inside the cell wall that surrounds the septum ring through a still unknown mechanism (de Medina-Redondo et al., 2010). Our analysis suggests that the 6Cys-box of the *Schizosaccharomyces* Gas2 proteins could function as a β(1,3)-glucan binding module that keeps the protein attached to the septum ring. Future experiments need to be addressed to verify this hypothesis.

The delta proteins of Pezizomycotina are very similar to *Schizosaccharomyces* Gas2 (Fig. 2), thus we suggests that they could have arisen from a common ancestor through complete loss of the Cys-box and significant divergence of the GH72 domain from that of the other subfamilies (note the differences in the catalytic bipartite motif described in the Results). The identification of this new subfamily can stimulate further investigation on the specific function of the GH72 delta proteins (i.e., Gel6 of *A. fumigatus*).

A further remarkable novelty of this study is that only one GH72 protein per species, always containing a 8Cys-box, has been found in the complete genome of each investigated Basidiomycota outgroup species, defined on the basis of a phylogenomics fungal tree (Medina et al., 2011). These outgroups consist of five Basidiomycota species belonging to the three subphyla of Pucciniomycotina, Ustilagomycotina and Agaricomycotina (see Section 2 and Supplemental Table S1). Although we cannot exclude a failure to identify other Basidiomycota GH72 proteins, due to partial genome sequencing or mis-annotation, these data lead us to hypothesize that GH72^{+} could be the most ancient GH72 subfamily. Consequently, the Ascomycota GH72^{-} subfamily could have been

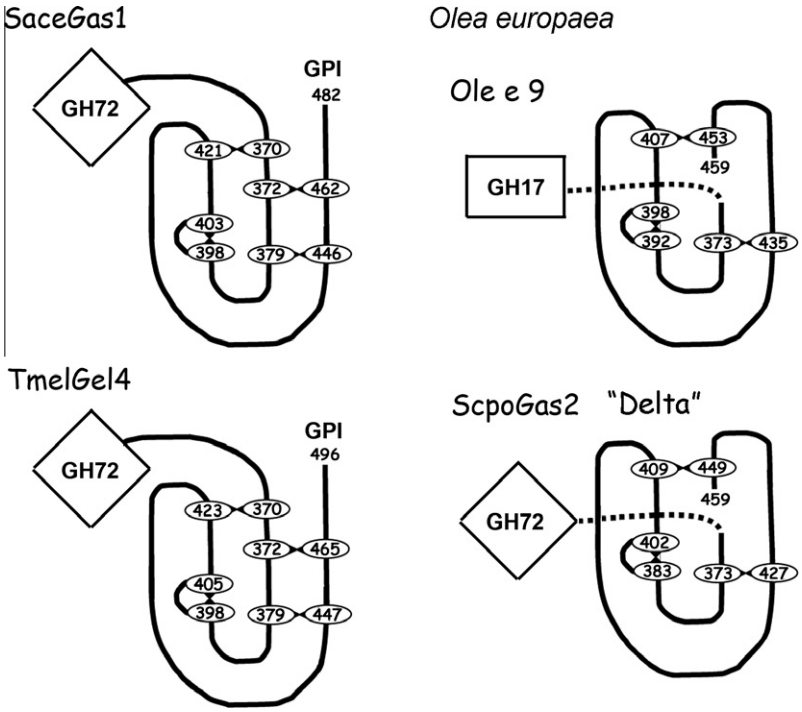

Fig. 4. Disulfide bond structure of the Cys-box in fungal β(1,3)-glucanosiltransferases (GH72^{+}), and plant GH17 β(1,3)-glucanases. The Cys residues of the Cys-box are shown as numbered ovals. **SaceGas1:** Experimentally determined disulfide bond network of the Cys-box in *S. cerevisiae* Gas1p (Popolo et al., 2008; Hurtado-Guerrero et al., 2009). **TmelGel4**: Network of potential intra-domain disulfide bonds in the Cys-box of *TmelGel4*. **Ole_e_9:** Experimentally determined network of the 6Cys-box appended to the GH17 glucanase domain of the olive pollen "Ole e 9" (Palomares et al., 2003). **ScpoGas2**: Network of potential disulfide bonds in *S.pombe* Gas2 protein as deduced from the plant 6Cys-Box.

originated from the secondary loss of the Cys-box from a GH72^{+} ancestor, and the delta paralogs could be a transition form from the GH72^{+} to the GH72^{-} subfamily. This hypothesis is also supported by the observation that, unlike GH72^{-}, most GH72^{+} genes are essential genes for vegetative cells or spores. In fact, we could speculate that, in a wide multigene family such as GH72, the original gene function, the essential one, has been retained by the most ancient members, while new functions have been acquired by most recent duplicated gene copies.

With respect to the two GH72 subfamilies defined by Ragni et al. (2007), we have here described in details several new evolutionary peculiarities and paralogous proteins for each of these subfamilies. In fact, GH72^{+} is characterized by numerous recent gene duplications that have originated a plethora of paralogous and taxon-specific GH72^{+} proteins: the G1 and G2 paralogs of Saccharomycotina; the two different G1 paralogs of a not yet well-defined Saccharomycotina subgroup; the four distinct GH72^{+} paralogs of Pezizomycotina Eurotiales (Fig. 2 and Supplemental Fig. S2). According to this evolutionary pattern, only one member of the GH72^{+} subfamily is present in *T. melanosporum.*

As for the GH72^{-}, this subfamily is characterized by the ancient gene duplications that gave rise to the three paralogous alpha, beta and gamma proteins, as well as by some gene losses (Fig. 2 and Supplemental Fig. S3). Remarkably, the gamma protein is present only in Saccharomycotina and in *T. melanosporum* (*TmelGAS4*), but in no other Pezizomycotina species. This suggests that the function of *TmelGAS4* is in some way related to the particular features of *Tuber*, which has a complex life cycle with the formation of hypogeous FBs. Thus, more extensive studies are needed to obtain a precise characterization of the function of the gamma GH72^{-} proteins and, in particular, of *TmelGAS4.* Finally, the GH72^{-} alpha paralog is characterized by at least two distinct gene duplication events, likely occurred one in the ancestor of *Schizosaccharomycetes* species and the other in the ancestor of only three main lineages of Pezizomycotina (Supplemental Fig. S3). According to this evolutionary pattern, *T. melanosporum* has only one alpha and one beta GH72^{-} protein.

Although the roles of some GH72 genes have been determined, at least in part, and mostly in yeast and human pathogenic fungi, their function during fungal development and morphogenetic processes (i.e., FB formation) still need to be investigated. The transition from vegetative mycelium to reproductive stage in truffles requires differentiation processes that lead to edible FBs consisting of different cell and tissue types. Several genes involved in cell wall biogenesis/degradation are finely regulated during the formation of a complex structure such as the FB (Lacourt et al., 2002; Poeggeler et al., 2006; Busch and Braus, 2007). In a previous paper on *Tuber borchii*, *in situ* hybridization experiments have demostrated that different chitin synthase genes are expressed differentially in FBs: *Tbchs3* appears to be involved in spore maturation, whereas *Tbchs4* may play a role in ascomata enlargement (Balestrini et al., 2000). Together with chitin, β(1,3)-glucan has been detected on the hyphal walls of truffles in several life cycle stages (Balestrini et al., 1996, 2012). In the FBs of *T. melanosporum*, labeling with anti β(1,3)-glucan antibodies was very abundant on the thick electron-transparent ascus wall (in addition to the hyphal wall) and gold granules were detected on the spore wall in the more internal region (data not shown).

Among the *T. melanosporum* genes involved in β(1,3)-glucan remodeling, array data have shown that *TmelGEL1* was the gene with the highest up-regulation in FB with respect to FLM (Table 2), although *TmelGEL1* had a very low expression level in the mycelium. The validation of these data by Real-Time qRT-PCR confirmed that *TmelGEL1* is the most up-regulated GH72 gene in FB *versus* the mycelium. Thus, *TmelGEL1* could play a role in the FB formation. However, in the internal region (gleba) of truffle FB there are both reproductive structures (asci containing the ascospores) and vegetative hyphae, which are distributed among the asci and also at the

periphery of the gleba (Balestrini et al., 2000). LMD was used to monitor the expression of these genes in these two different compartments of the FB. While *TmelGEL2*, *TmelGEL4* and *TmelGAS4* were expressed in both homogeneous cell-type populations, suggesting a functional redundancy in these genes, *TmelGEL1* transcript was detected only in the vegetative hyphae of the FB, suggesting a specific role of *GEL1* inside this multigene family. *TmelGEL1* could be involved in the remodeling of cell wall β(1,3)-glucan during hyphal growth and aggregation in FB, rather than in the differentiation of the sexual structures.

It has been shown that GH72 enzymes play partially overlapping roles throughout the fungal life cycle (Ragni et al., 2007; Gastebois et al., 2010; Rolli et al., 2011). In *A. fumigatus*, *GEL1*, *GEL2* and *GEL4* are constitutively expressed during mycelium growth and, out the 3 genes, *GEL4* is the most expressed during growth and it is also essential for this fungus (Gastebois et al., 2010). Previous experiments have also shown that the *gel1* deletion mutant has no effect on phenotype, while the Δ*gel2* and Δ*gel1*Δ*gel2* mutants exhibit a reduced growth, abnormal conidiogenesis and a decrease in virulence (Mouyna et al., 2005).

We explored the ability of *TmelGEL1* to complement the phenotypic defects of the *gas1*Δ mutant of *S. cerevisiae* (Popolo et al., 1993; 1997). In other works this approach proved successful to assess the similarity of the function of GH72 enzymes from different yeast and fungal species. For instance, *PHR1* from *C. albicans* complements *gas1*Δ mutation (Vai et al., 1996) and *AfGEL1* fully complemented, and *A. fumigatus GEL2* partially complemented, *S. cerevisiae gas1*Δ phenotype (Mouyna et al., 2005). In all these studies, the *S. cerevisiae GAS1* signal peptide was fused to the heterologous ORFs. On the contrary, the full-length *TmelGEL1* cDNA failed to restore the phenotypic defects of the yeast mutant. Before concluding that the protein plays a different function, other explanations should be taken into account such as inefficient translocation in the ER or folding, with consequent protein degradation. Alternatively, *Tmel*Gel1 may be a protein difficult to produce when its level of expression is artificially raised, as previously observed for Gas3p of *S. cerevisiae* (Rolli et al., 2010). Moreover, even other genes encoding GH72 enzymes from filamentous fungi, such as *Bbgas1* of *Beauveria bassiana* and *gas1* of *Fusarium oxysporum*, did not complement the phenotype of the *S. cerevisiae gas1*Δ mutant (Zhang et al., 2011; Caracuel et al., 2005). The availability of antibodies that specifically recognize the proteins, and further biochemical studies will be useful in assessing this issue.

In conclusion, our data increase the knowledge of β(1,3)-glucanosyltransferases, by adding new information on this enzyme family in a Pezizomycetes symbiotic fungus. The results suggest that several members of this family show a partial functional overlap, both in the mycelium and in the edible FB. Further biochemical analyses, including *in vitro* GH72 activity assays and characterization of the enzyme properties, will be essential to understand how *Tmel*Gel/Gas participate in cell wall biogenesis and cross-linking during the morphogenetic program of *T. melanosporum*. Indeed, the structure of glucan fibers, the type of wall components and intracellular/extracellular environmental conditions (pH, ion concentrations) may differ during developmental transitions and require the contribution of different GH72 enzymes with peculiar biochemical properties (Mazáň et al. 2011).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.01.010.

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