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The GH72 family of Tuber melanosporum is composed of four genes (TmelGEL1, TmelGEL2, TmelGEL4 and TmelGAS4). TmelGEL1 expression is up-regulated in fruiting body (FB) compared to the free mycelium. Inside the FB, TmelGEL1 expression is restricted to the hyphae, which were isolated by laser microdissection. The phylogenetic tree in 51 Ascomycota identifies 3 subfamilies (GH72+/- and delta). Taxon-specific paralogous genes are identified.
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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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 mycorrhiza 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 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 (CHS2, CHS3, CHS4, CHS5 and CHS6), 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 RhodGTPase subunit (RhodA), 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/transglycosylase activity. In vitro, they internally cleave a laminarooligosaccharide of >10 glucose residues and then transfer the newly generated reducing end to the non-reducing end of another laminarooligosaccharide, 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, PHA4 and PAG5). 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, ScGas1 and ScGas5 proteins are required for cell wall formation during vegetative growth, with Gas2 playing the major role while Gas5 only has an auxiliary function. ScGas2 and ScGas4 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 Norkrans (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 and quantitative Reverse Transcriptase (RT)-PCR methods were 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-blast/index.cgi). Prior to Real-Time qRT-PCR, the primers were
tested on genomic DNA (Balestrini et al., 2012). Real-time qRT-PCR was carried out with 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 °C 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 ratio was calculated without the PCR efficiency correction from equation 2,\[ \Delta CT = \Delta CT_{control} \] where \( \Delta CT \) represents the \( \Delta CT \) sample \( \cdot \Delta CT \) control. All the reactions were performed for at least two biological and three technical replicates. Statistical analyses were carried out using R version 2.10.2, 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 into the pGEM-T plasmid (Promega) using T4 DNA Ligase (Promega). The 10 μl reaction mix 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 MicroPulsar™ 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.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 [Ethyol: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 800g 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 replicates and two technical replicates.

### 2.6. Phylogenetic analyses

We analyzed the GH72 proteins of the three major groups of Ascomycota (Saccharomyceota, 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 Saccharomyces 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, Mal Replay (http://mendel.imp.ac.at/METH-ODS/seg.server.html). The Ser/Thr-rich domains were defined as having a high Ser/Thr content. The presence and location of signal sequences were used as outgroup taxa in the analysis of the to-
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, *TmeI-Gel4* 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), *TmeI-Gel4* belongs to the GH72+ subfamily, while *TmeI-Gel1*, *TmeI-Gel2* and *TmeI-Gas4* 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., 2008; 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 (1,3)-glucan binding domain appended to a catalytic module (Palomares et al., 2003).

The multi-alignment of the *TmeI* 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 *TmeI* 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 Saccharomyces 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 Saccharomyces 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 Basidiomycotina 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).

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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 (striped 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 (154-VFANETYV and 254-FTSFEGC), in Gel2 (153-VFANETYV and 257-FTSFEGC), in Gas4 (154-FLAGNEVIF and 257-FITFYEG) and Gel4 (154-FLAGNEVIF and 257-FTSFEGY). The tyrosines of the active site are marked by a star. The arrow marks the predicted GPI-attachment site in Gel1: G494–493, in Gel2: G474–473, in Gas4: G495, and in Gel4: 419–425. 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.

Each *Tmel* proteins lacking the Cys-box (*Tmel*Gel1, *Tmel*Gel2 and *Tmel*Gas4) is located in a distinct clade of the GH72 subfamily. These clades are highly supported (bootstrap values > 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* (Pezizomyces, Pezizales) is the basal branch of Pezizomyces, with Sacharomyces sister taxon of Pezizomyces, 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 Saccharomyces species and *TmelGEL4* as the sole Pezizomyces representative. Since the evolutionary tree in Fig. 2 includes the full GH72 complement of 24 Pezizomyces and 2 *Schizosaccharomyces* 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 Pezizomyces 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 Saccharomyces and Pezizomyces, 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 Pezizomyces 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 Pezizomyces clade (including *TmelGel4*); (b) two distinct Saccharomyces clades which have been named G1 and G2, since they comprise *S. cerevisiae* Gs1 and Gs2, respectively. Unfortunately, the evolutionary history of the GH72 subfamily cannot be fully reconstructed because most of the internal nodes are unresolved (bootstrap values < 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 Saccharomyces clades: (a) a GH72 gene duplication occurred only in the ancestor of Saccharomyces; or (b) a GH72 gene duplication occurred in the common ancestor of Saccharomyces and Pezizomyces, followed by gene loss in Pezizomyces. 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 Gs2 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 Pezizomyces classes. Thus, the delta protein is absent in the fifth Pezizomyces lineage to which the *Tuber melanosporum* belongs (Fig. 2 and Supplemental Fig. S3). The delta proteins have some unusual features. In particular, the *Schizosaccharomyces* Gs2 proteins show: (1) a modified Cys-box domain (named Gcys-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* Gs2 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 Pezizomyces have neither a standard Cys-box (8Cys-box) nor a modified Gcys-box, and show a modified bipartite motif surrounding the two conserved catalytic Glu residues (Fig. 1B). Indeed, among all GH72 proteins only the delta Pezizomyces have the (V/Y)CNE and the SETG sequences around the catalytic Glu. Thus, there is a conservative A → 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 Pezizomyces (for example, *A. fumigatus* Gel6 of Fig. 2). It should also be noted that the basal polytomies of our phylogenetic tree and the fairly small resolution of the GH72 clade 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 Saccharomyces 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 Pezizomyces (Eurotiales), giving rise, among others, to the four proteins of *A. fumigatus* (Asfl Gel3, Gel4, Gel5, and Gel7). These duplications certainly occurred after the separation of Eurotiales from Omygenales, as there is a single GH72 protein in the genomic sequence of the three analyzed Omygenales 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 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 Pezizomyces classes. On the contrary, in GH72 the most ancient duplication event originated the G1 and G2 paralogs of Saccharomyces (this event cannot be exactly mapped because of the limited tree resolution), but three more recent duplications occurred in Saccharomyces (G1 group) and in Pezizomyces Eurotiales.

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

It was previously observed that members of the (1,3)glucan-syltransferase 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-mentioned 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 TmelEF1B (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_002640160.1) 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 hyphal compartment. In addition, TmelGEL1 clusters in the alpha clade (Fig. 2) together with A. fumigatus Gel1 that is able to completely 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 TmelGEL1 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 (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.
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 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 Schizosaccharomyces 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 Schizosaccharomycetes 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 Pezizomycotina 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 motif described in the Results). The identification of this new subfamily could function as a (Cys-box) (de Medina-Redondo et al., 2010). Our analysis suggests that the 6Cys-box of the Schizosaccharomycetes 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 Schizosaccharomycetes Gas2 (Fig. 2), thus we suggest 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 (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 Schizosaccharomyces proteins (see dashed blocks in Fig. 2), while in Pezizomycotina the C-terminal LC region shows a highly variable Ser/Thr content.

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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 Saccharomyces and in T. melanosporum (TmelGEL4), but in no other Pezizomycotina species. This suggests that the function of TmelGEL4 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 TmelGEL4. Finally, the GH72 alpha paralog is characterized by at least two distinct gene duplication events, likely occurred one in the ancestor of Schizosaccharomyces 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 demonstrated 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 ascocoma 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

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In conclusion, our data increase the knowledge of what will be useful in assessing this issue. For example, in T. melanosporum (Gastebois et al., 2010; Rolli et al., 2011), it has been shown that GH72 enzymes play partially overlapping roles in the differentiation of the sexual structures. While glucan during hyphal growth and aggregation in FB, rather than 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, GEI, GE2 and GE4 are constitutively expressed during mycelium growth and, out of the 3 genes, GE4 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 Age2 and Age1Age2 mutants exhibit a reduced growth, abnormal conidogenesis and a decrease in virulence (Mouyna et al., 2005).

We explored the ability of TmelGEI to complement the phenotypic defects of the gas1A mutant of S. cerevisiae (Popolo et al., 1993; 1997). In other words, 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 gas1A mutation (Vai et al., 1996) and AfgE1 fully complemented, and A. fumigatus GEI2 partially complemented S. cerevisiae gas1A 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 TmelGE1 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, TmelGE1 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 gas1A 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)-glucan synthases, 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 vitroGH72 activity assays and characterization of the enzyme properties, will be essential to understand how TmelGal/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.

5. United references

Carotti et al. (2004), Hartland et al. (1996), Mazâa et al. (2011), and Wessels and Sietsma (1979).

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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/10.1161/j.fgb.2013.01.010.


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