**Hyphal and cytoskeleton polarization in Tuber melanosporum: A genomic and cellular analysis**

# Hyphal and cytoskeleton polarization in *Tuber melanosporum*: A genomic and cellular analysis

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## Abstract

Filamentous polarized growth involves a series of events including polarization of the cytoskeleton to selected growth sites, and the transport of secretory vesicles containing the components required for growth. The availability of fungal genome sequences has recently led to the identification of a large number of proteins involved in these processes. We have explored the *Tuber melanosporum* genome sequence by searching for homologs of genes known to play crucial roles in the morphogenesis and cell polarity of yeasts and filamentous fungi. One hundred and forty-nine genes have been identified and functionally grouped according to the deduced amino acid sequences (44 genes involved in cell polarity/morphogenesis, 39 belonging to the actin cytoskeleton and 66 involved in membrane dynamics, septation and exocytosis). A detailed gene annotation has shown that most components of the cell polarity machinery, morphogenesis and cytoskeleton found in yeasts and filamentous fungi are conserved, although the degree of similarity varies from strong to weak. Microscopic analysis of quick-frozen truffle hyphae detected the characteristic subcellular components of the hyphal tip in septate filamentous fungi, while transcript profiles revealed a moderately variable pattern during the biological cycle.

## Keywords

- *Tuber melanosporum*;
- Cytoskeleton;
- Fungal morphogenesis;
- Polarized growth;
- Transcript profile;
- Freeze substitution

# 1. Introduction

Filamentous fungi grow by means of apical expansion of tube-shaped cells (i.e., hyphae). Hyphal tip growth is characterized by the initial establishment of one growth site, followed by its continuous maintenance. This differs from budding in yeasts, which is characterized by a short period of polarized growth followed by isotropic growth that leads to an almost spherical daughter cell (Steinberg, 2007a). The newly available genome sequencing of many fungi (NCBI) has highlighted informative homologies between the *Saccharomyces cerevisiae* yeast and filamentous fungi. Several components of the polarisome multiprotein complex, which regulates microfilament formation at polarized growth sites in yeast, are well conserved in the filamentous fungal genomes, such as *Neurospora crassa* and *Aspergillus nidulans* ( Harris and Momany, 2004, Steinberg, 2007a and Steinberg, 2007b). However, hyphal apical growth requires additional events, such as the addition of new material and the coordination of a localized deposition.

Several investigations have suggested that the Spitzenkörper, a vesicle-rich region that defines the center and direction of growth, and other proteins may play a crucial role in such a polarized hyphal extension (Bartnicki-Garcia, 1990, Harris et al., 2005, Roberson et al., 2009 and Wessels and Simpson, 2007). Some of the proteins involved in these processes belong to the polarisome complex, which promotes polarized morphogenesis through the regulation of the actin cytoskeleton and signaling pathways (Sheu et al., 1998). Polarity allows vesicles to flow in the direction of the Spitzenkörper prior to their fusion with the plasma membrane in order to provide the precursors and enzymes that are required for cell extension and nutrition.

Other aspects of particular interest in filamentous fungi include the mechanisms that are at the basis of cell elongation, the formation of new branches and their regulation. It has been demonstrated that such morphogenetic changes depend on cytoskeleton modifications and are triggered by external or intracellular signaling (Bassilana et al., 2005, Lee and Kronstad, 2002, Thomas et al., 2003, Uetake and Peterson, 1997 and Weber et al., 2005). Such a complex network is likely elicited by the recognition of a spatial landmark, which triggers the aggregation of protein complexes which – in turn – initiate the assembly and polarization of cytoskeleton components that eventually direct vesicle delivery to growth sites (Finger et al., 1998).

The aim of this paper was to explore the *Tuber melanosporum* genome sequence by searching for gene homologs known to play crucial roles in both morphogenesis and/or polarity establishing and maintenance in yeasts ( Drees et al., 2001) and in filamentous fungi ( Banuett et al., 2008 and Borkovich et al., 2004).

The Perigod black truffle *T. melanosporum* is an ascomycetous ectomycorrhizal fungus that belongs to the *Pezizales* order. Truffles, like some other ectomycorrhizal fungi, have a dual lifestyle, living both in the soil as facultative transitory saprotrophs and within the plant roots as symbionts ( Martin and Nehls, 2009). This duality is reflected on the alternation between the hyphal life, which leads to the formation of ectomicorrizae (ECM) and symbiosis, and the fructification stage, which leads to the formation of fruitbodies containing sexual spores. During these transitions, the mycelium undergoes morphogenetic changes, caused by the expression of specific genes, which are triggered by genetics and environmental factors ( Martin and Nehls, 2009 and Martin et al., 2010).

The examined gene/protein networks were divided on the bases of the roles of the single components, that are known in other fungi (*S. cerevisiae*, and other filamentous fungi; Banuett et al., 2008; Borkovich et al., 2004; Harris et al., 2009).

The first considered group of genes/proteins was represented by the Rho GTPases and Cdc42 modules, small Rsr and several effectors. Actin and tubulin cytoskeleton components were also examined, together with their upstream regulators: the GTPase signaling pathway, via Wiskott–Aldrich syndrome protein (WASP)-like, Sla proteins and other elements that lead to actin nucleation (Drees et al., 2001 and Pollard, 2003). Finally, proteins that belong to the exocyst, polarisome and Spitzenkörper complexes were considered.

# 2. Materials and methods

## 2.1. Biological materials

The haploid monokaryotic line Mel28 of *T. melanosporum* was obtained, by Chantal Dupre and Gerard Chevalier, from a fruitbody collected by Louis Riousset at St. Remy de Provence (Bouches-du-Rhone, France) and deposited at the INRA-Clermont-Ferrand. Ectomycorrhizae (ECMs) of *T. melanosporum* were sampled from mycorrhized hazelnut plants (*Corylus avellana*), produced by AGRI-TRUFFE (Saint-Maixant, France). *T. melanosporum* fruitbodies were collected below common hazel trees or oak trees at different locations ( Martin et al., 2010).

## 2.2. In silico genome automatic annotation and manual curation

The putative genes (gene models) that encode the proteins and enzymes involved in hyphal growth and polarization were searched for and identified in the *T. melanosporum* Mel28 genome, using BLAST and Gene Ontology tools at the TuberDB *Tuber* genome database (http://mycor.nancy.inra.fr/IMGC/TuberGenome/). Additional searches were performed by launching a range of specific genes that are available from fungi at the NCBI GenBank (http://www.ncbi.nlm.nih.gov) and UNIPROT (http://expasy.org/) to the *Tuber* genome database, using BLASTN, TBLASTN, and BLASTP algorithms.

All the detected gene models were inspected manually, and automatically selected gene models of the TuberDB database were modified when necessary. The putative orthologous that were detected were characterized on the basis of the conserved domains, identities, and *E*-values. Manual annotation was carried out using the Artemis software (http://www.sanger.ac.uk/Software/Artemis/), taking into consideration Expressed Sequence Tags (ESTs), when available. The manually annotated gene sequences were aligned using the CLUSTALX programme (version 1.83.1) (Jeanmougin et al., 1998). Each curated ortholog was also used for a BLAST search at http://mycor.nancy.inra.fr/IMGC/TuberGenome/blast.php, which includes a database with five reference ascomycota: *N. crassa*, *Magnaporthe grisea*, *Botrytis cinerea*, *A. nidulans* and *S. cerevisiae*.

## 2.3. Phylogenetic analysis

The deduced amino acid sequences were used for extensive database searching for homolog sequences. Protein sequence data were taken from SWISS-PROT and EMBL protein databases and the GenBank non-redundant protein database. A multiple protein alignment was applied using the ClustalX package (Thompson et al., 1997). Phylogenetic trees were constructed by means of the neighbor joining (NJ) method (Saitou and Nei, 1987), using the Kimura 2-parameter model and the MEGA computer programme, Version 4.046 (Kumar et al., 1994). Bootstrap analyses were based on 1000 re-samplings of the sequence alignment.

## 2.4. Transcriptome analyses

Transcript profiling was carried out comparing free-living mycelium (FLM) grown on a glucose-rich agar-medium with ectomycorrhizal root tips of *T. melanosporum–Corylus avellana*, and fruiting bodies (FB), as described in Martin et al., 2010. The *T. melanosporum* custom-exon expression array (4 × 72 K) manufactured by Roche NimbleGen Systems Limited (Madison, WI) contained five independent, nonidentical, 60-*mer* probes per gene model coding sequence. 7496 annotated protein-coding gene models, 5736 TE sequences, 3913 random 60-*mer* control probes and labeling controls were included in the oligoarray. Technical duplicates were included on the array for 1876 gene models. The total RNA preparations (four biological replicates for ectomycorrhizae, five for fruiting bodies and seven for free-living mycelium) were amplified using the SMART PCR cDNA Synthesis Kit (Clontech), according to the manufacturer's instructions.

Single dye labeling of the samples, hybridization procedures, data acquisition, background correction and normalization were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland), according to their standard protocol.

Raw array data were filtered for non-specific probes (a probe was considered non-specific if it shared more than 90% homology with a gene model other than the gene model it was made for) and renormalized using the ARRAYSTAR software (DNASTAR, Inc. Madison, WI, USA). No reliable probe was left for 1015 gene models. A transcript was deemed to be expressed when its signal intensity was threefold higher than the mean signal-to-noise threshold (cut-off value) of 3913 random oligonucleotide probes present on the array (50–100 arbitrary units). Gene models with expression values higher than threefold the cut-off level were considered as transcribed. A Student *t*-test with false discovery rate (FDR) (Benjamini-Hochberg) multiple testing correction was applied to the data using the ARRAYSTAR software (DNASTAR). Transcripts with a significant *p*-value ($<0.05$) and $\geqslant$fourfold change in the transcript level were considered as differentially expressed. The complete expression dataset is available as a series (accession number GSE17529) at the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/).

Expression levels of the annotated genes were also detected through massive cDNA sequencing of the aforementioned FLM, ECM and FB libraries with the Solexa/Illumina technology. Deep sequencing was carried out at the Genoscope facilities, as described in Denoeud et al. (2008) and mapped to the *T. melanosporum* genome and gene models, as reported by Martin et al. (2010). The single-end reads obtained were 36 nucleotides long and were deposited in Tuber Gbrowse (http://mycor.nancy.inra.fr/cgi-bin/secure/gbrowse/). The data were normalized according to sample size and gene length and reported in Reads per kilobases of exon model per million mapped reads RPKM).

## 2.5. Transmission electron microscopy

Fungal stock cultures were maintained on a malt extract medium. Autoclaved dialysis membranes were placed on the surface of malt agar in 9 cm Petri dishes, in preparation for cryofixation. Small portions of actively growing mycelia were transferred to the dialysis membrane surfaces and the cultures were grown at 25 °C. After 15 days, the membrane surfaces were well colonized. The hyphal monolayers and supporting membranes were trimmed with a razor blade to approximately 4 × 5 mm, and left undisturbed on the agar surface for 45–60 min. Samples were then quick-frozen by plunging them into liquid nitrogen-cooled propane (Hoch, 1986 and Howard and O'Donnell, 1987) and subsequently processed for freeze substitution and embedding, as described in McDaniel and Roberson (2000). After cryofixation, the samples were freeze substituted at −85 °C in 1% glutaraldehyde (Electron Microscopy Sciences, Washington, PA, USA) and 1% tannic acid in

anhydrous acetone for 72 h. The samples were then rinsed thoroughly with three changes of anhydrous acetone at −85 °C for a total of 45 min. The cells were transferred into 1% $OsO_4$ in acetone for 1 h at −85 °C before being slowly warmed, over 2 h, to −20 °C, then to 4 °C over an additional 2 h, and finally to room temperature over 1 h. The specimens were washed with three changes of anhydrous acetone for a total of 45 min, slowly infiltrated with Epon–Araldite resin and flat embedded between two Teflon coated glass slides (Hoch, 1986 and Howard and O'Donnell, 1987). Well-frozen hyphae were selected and prepared for thin sectioning according to Howard and O'Donnell (1987). Ultra thin sections were post-stained for 5 min in 2% uranyl acetate in 50% ethanol and 5 min in Reynolds lead citrate (Reynolds, 1963). The sections were examined under a Philips CM10 transmission electron microscope in the Laboratory of Advanced Microscopy, Department of Plant Biology at the University of Turin.

# 3. Results

In this study we explored the genome sequence of *T. melanosporum* to search for orthologs. The components that are present and conserved in the *T. melanosporum* genome are described hereafter together with those that are either absent or highly diverged ( Tables 1S and 2S).

## 3.1. Rho-type GTPases as key regulators of polarity

Rho GTPases are key regulators of the actin cytoskeleton; it has been shown that they influence an amazing variety of cellular processes that are crucial for coordinated morphogenesis. *S. cerevisiae* contains one Cdc42 and five Rho proteins, from Rho1 to Rho5 ( Borkovich et al., 2004 and Etienne-Manneville and Hall, 2002; Van Aelst and D'Souza-Schorey, 1997). The *T. melanosporum* genome contains one Cdc42 and four Rho proteins, while Rho5 (present in yeast) is absent, as in other filamentous fungi, such as *A. nidulans* and *N. crassa* ( Borkovich et al., 2004, Etienne-Manneville and Hall, 2002, Harris, 2006 and Harris et al., 2009). The *T. melanosporum* genome contains a clear ortholog of Rac, which is conserved in filamentous fungi and higher eukaryotes, but is missing in all sequenced yeasts (*S. cerevisiae*, *Saccharomyces pombe*, and *Candida albicans*). Rac proteins can play a crucial role in actin-dependent polarized growth and division, and its absence can inhibit the switch to filamentous growth in dimorphic fungi ( Boyce et al., 2003).

Furthermore, a new Rho1-like protein (GSTUM00001369001) was found in the *T. melanosporum* genome. The protein has a clear Rho domain and shows a high similarity with the Rho1 cluster, as shown in the phylogenetic tree ( Fig. 1). A comparison of Rho genes and/or deduced amino acid sequences was performed (Fig. 1S). The *T. melanosporum* Rho proteins are around 200 residues long, which is in the range of almost all small GTPases. The alignment allowed us to highlight the key conserved regions involved in nucleotide and Mg ion binding, which are conserved in all Rho GTPases, the Rho insert and the characteristic CAAX motif immediately preceded by a polybasic domain rich in lysine residues (K).

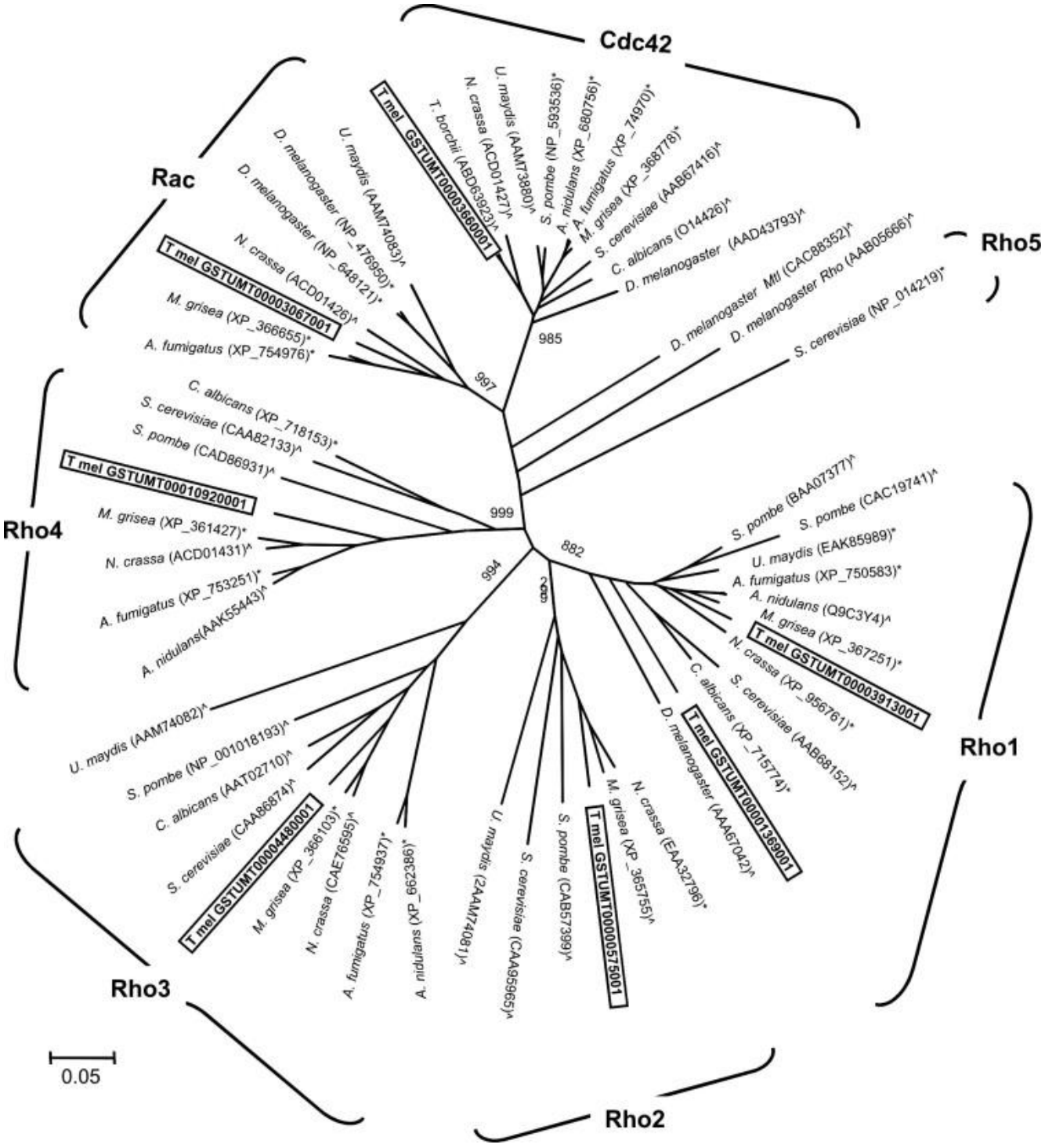


Fig. 1. Phylogenetic relationships between the filamentous ascomycetes (*Aspergillus fumigatus, Aspergillus nidulans, Candida albicans*, *Magnaporthe grisea, Neurospora crassa, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Tuber borchii),* Basidiomycetes (*Ustilago maydis*), and Metazoa (*Drosophila melanogaster*) based on amino acid deduced sequences derived from mRNA (*) and protein sequences (^) for representative RHO family. The unrooted tree was constructed using the neighbor-joining method. Sequences were manually annotated and analyzed using Clustal W. The percentages at the nodes represent levels of bootstrap support from 1000 resampled datasets. GenBank accession numbers are given in parentheses. The *T. melanosporum* sequences analyzed in this study are highlighted.

A phylogenetic analysis of the *T. melanosporum* Rho proteins and the orthologous fungal sequences encoding for Rho1, Rho2, Rho3, Rac and Cdc42 was performed and the results show that *T. melanosporum* Rho proteins fall into distinct subgroups ( Fig. 1). Bootstrap analysis supported the monophyletic clusters obtained within the Rho subfamilies. However Rho5 from *Saccharomyces* resulted radiale from the other Rho groups without apparent phylogenetic relatives. This observation is in agreement with the phylogenetic affiliation of Rho-related proteins described by Rivero et al., 2001. Members of the Rho2, Rho3, Cdc42 and Rac groups are evolutionarily conserved, while the available sequences constituting the Rho4 class are quite strongly diverged, making it difficult to predict potential functions.

### 3.2. Exocyst complex proteins, polarisome and Spitzenkörper elements

The exocyst complex comprises eight proteins: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, involved in the docking of exocytic vesicles to the growing tip of the cellular membrane, an indispensable event for hyphal-cell growth, in that it permits the deposition of the material necessary for cell wall synthesis and plasma membrane extension (TerBush et al., 1996 and Ram et al., 2002). The *T. melanosporum* genome contains coding information on all the components of the exocyst complex, as well as two copies of Sec10 and Sec15 each.

The polarisome was identified in *S. cerevisiae* as a 12S multiprotein complex that contains Spa2, Pea2, Bni1 and Bud6/Aip3 and it was proposed that this complex promotes polarized morphogenesis through the regulation of the actin cytoskeleton and signaling pathways. The signal from different Rho proteins in fact converges on the Bni1 formin, a polarisome component, to control actin organization ( Sheu et al., 1998). The components Bni1, Spa2, Bud6-Aip3 of the polarisome complex were found in the *T. melanosporum* genome, while Pea2 (present in *S. cerevisiae*, *Pichia stipitis*) was absent**,** as in *Ustilago maydis* ( Banuett et al., 2008), *A. nidulans* ( Harris and Momany, 2004) and *N. crassa* ( Borkovich et al., 2004).

Recent work on different filamentous fungi indicates that formin, myosin light chain, Sec4 (a Rab GTPase), BemA (homolog of *S. cerevisiae* Bem1), TeaA and TeaR (homologs of *S. pombe* end cell markers Tea1 and Mod5, respectively), and two chitin synthases are components of the Spitzenkörper ( Crampin et al., 2005, Leeder and Turner, 2008 and Riquelme et al., 2007). The *T. melanosporum* genome contains coding information on all of these components except TeaR (Mod5).

### 3.3. Actin cytoskeleton and polar growth proteins

The actin cytoskeleton in *S. cerevisiae* is involved in polarized secretion. Actin cables serve as tracks on which vesicles move on myosin motors towards active growth and secretion sites (the bud tip during polarized growth and the neck region during cytokinesis) ( Pruyne and Bretscher, 2000a, Pruyne and Bretscher, 2000b and Xiang and Plamann, 2003).

The *T. melanosporum* genome did not offer any major surprises at the structural components of the cytoskeleton. Homologs of proteins, such as actin binding protein (Abp1), coronin (Crn1), profilin (Pfn), and an assembly of cortical actin cytoskeleton proteins (Sla1 and Sla2), fimbrin (Fim), Pan1, some of which are actin patch components, were found in the *T. melanosporum* genome. One formin was found, while 2 and 3 are present in budding and fission yeast, respectively ( Pruyne et al., 2002). A Las1-like protein with no match to *S. cerevisiae* Las1 was also found. This protein is highly variable compared to the Las1-like one detected in *N. crassa*, *B. cinerea* and *M. grisea*.

Ten actin-related proteins (Arps) were found in *S. cerevisiae*, and *T. melanosporum* encodes all of them except Arp7, Arp9 and Arp10 which are involved in chromatin remodeling. Genes that code for the Arp2–Arp3 complex ( Winter et al., 1999), which are responsible for the nucleation of branched actin structures, are present in the *T. melanosporum* genome. One gene coding for the Bee1/Las17 (homolog of the mammalian Wiskott–Aldrich syndrome protein WASP), a down-stream effector of Cdc42 and activator of Arp2/3, was identified, while Vrp1 (homolog of mammalian Wip, Wiskott–Aldrich syndrome protein interacting-protein) was absent. In *S. cerevisiae*, Arp2/3, Bee1/Las17 localize to actin patches and form an actin cap during early bud morphogenesis ( Pruyne and Bretscher, 2000a and Pruyne and Bretscher, 2000b).

The *S. cerevisiae* genome contains two tropomyosin isoforms, Tpm1 and Tpm2. These proteins stabilize actin cables ( Pruyne and Bretscher, 2000a and Pruyne and Bretscher, 2000b). The *T. melanosporum* genome contains a single tropomyosin gene, and the putative protein is similar to *S. cerevisiae* Tpm2, but its function in *T. melanosporum* still has to be determined.

Another component of the actin cytoskeleton is represented by myosins. Myosins are actin-dependent molecular motors that play important roles in contraction, cell motility, and organelle transport. As in other filamentous fungi, the *T. melanosporum* genome contains coding information on 4 myosins with a single member in each of the three classes found in *S. cerevisiae*: a class I TmelMyo5), class II (TmelMyhI), a class V (TmelMyo2) ( Tables 1S and 2S, Hodge and Cope, 2000).

In addition, *T. melanosporum* encodes two unconventional myosin domains linked to a chitin synthase domain, class XVII (TmelChs8 and TmelChs6).

A protein, called RmsA in *Aspergillus niger*, with a central role in several processes, such as polarization of actin at the hyphal tip, general metabolism, viability and stress resistance, was also found and annotated in *T. melanosporum*.

### 3.4. Genes involved in the tubulin cytoskeleton

The hyphal growth requires that a large amount of material is transported towards the hyphal tips for cell wall synthesis. Intracellular transport in budding yeast is exclusively myosin dependent. Filamentous fungi, like higher eukaryotes, instead utilize a combination of actin- and microtubule-based systems. Analyses of fungal genomes indicate that, in filamentous fungi, there are at least 10 distinct kinesins, which are the motor proteins that move along microtubule cables to support several cellular functions, such as mitosis, meiosis and transport of cargo (Schoch et al., 2003 and Rischitor et al., 2004; Steinberg, 2007c).

An examination of the *T. melanosporum* genome indicates that it encodes 10 distinct kinesins, 3 of which (Tmelklp1and Tmelklp2 both members of the kinesin-3 family and Tmelklp4 belonging to the kinesin-4 family) are likely to be involved in vesicle and organelle transport and DNA binding. Tmelklp4, in particular, shows a 47% identity with *N. fischeri*, which is related to members of the chromosome-associated kinesin KIF4. The proteins of this group seem to perform a variety of functions, and have been implicated in neuronal organelle transport and chromosome segregation during mitosis. The KIF4 group within the kinesin-4 family is conserved in higher plants, animals and in filamentous fungi, but it is not found in unicellular yeasts ( Schoch et al., 2003).

The blocked in mitosis C protein (Bimc, kinesin-5 family) and kinesin-like protein A (KlpA, kinesin-14 family) kinesins have been found to play roles in the mitosis of filamentous fungi and many other organisms. These proteins have also been identified in the *T. melanosporum* genome,

suggesting the existence of conservation in functions among filamentous fungi, higher eukaryotes and yeasts. However, in yeast, the BimC family members, Kip1p and Cin8p (kinesin-5 family), have at least partly overlapping functions, whereas in *T. melanosporum* only one mitotic kinesin is present. In addition to the Kip2p and Kip3p yeast ortholog genes (TmelKlp5, kinesin-7 family, and Tmelklp3 kinesin-8 family), the *T. melanosporum* genome contains two other kinesins (TmelKlp6 kinesin-10 family and TmelKlp7 kinesin-6 family). These genes are not present in yeast and are thought to be involved in the organization of the mitotic spindle and the chromosome alignment in the metaphase of animals and filamentous fungi. Their existence suggests different spindle formation mechanisms and functions between filamentous fungi, such as *T. melanosporum*, and unicellular yeasts.

The *T. melanosporum* genome was also analyzed to find dyneins, the motor proteins that convert the chemical energy contained in ATP into mechanical movement energy; they mediate various biological processes, including nuclear migration and organelle transport, by moving on microtubules while associated with various cellular structures. One heavy chain, one intermediate chain, one light intermediate chain and three distinct light chains dyneins, seven dynactin subunits, two components of the Lis1-complex (Lis1, Ro11, Borkovich et al., 2004) involved in dynein regulation and one NudC, were found in the *T. melanospourm* genome. After examination of the *T. melanosporum* genome, it was highlighted that the dynein/dynactin subunits of *T. melanosporum* are more similar to those of metazoans than to those of *S. cerevisiae*. Some of the dynein/dynactin subunits present in filamentous fungi and metazoans are not detectable in either *S. cerevisiae* or *S. pombe*: Dlic, Dlc, p150 Glued – nip100 (Tmel-ro3, dynamitin/p50, p62, p27, p25, and Tctex) ( Schroer, 2004). Dyn3 from *S. cerevisiae* is also absent.

Tubulin genes have been also found in the *T. melanosporum* genome. Three groups have been identified: two genes encoding the tubulin α-chain and two genes encoding the tubulin β-chain, in addition to a tubulin γ-chain gene, exactly as in *S. cerevisiae*, *A. nidulans* and other filamentous fungi ( Oakley, 2004). Likewise, one tubulin α-chain, two tubulin β-chain and one γ-chain genes are present in *U. maydis* ( Banuett et al., 2008).

Septins are cytoskeletal GTPase proteins which were first discovered in *S. cerevisiae* and are also present in animals. They are considered key players in cellular organization processes and have been shown to be important in fungi, for example, during septum formation and branching. Five candidate septins were identified in *T. melanosporum* data bank. All the five groups present in other fungi seem to be represented in the *T. melanosporum* with one member in each group ( Fig. 2). Another candidate (GSTUMT00012098001) was found in the *T. melanosporum* genome. However, only a small piece of the septin domain is present (position from 400 to 446) and the position is separate from the other sequences ( Fig. 2, Table 2S).

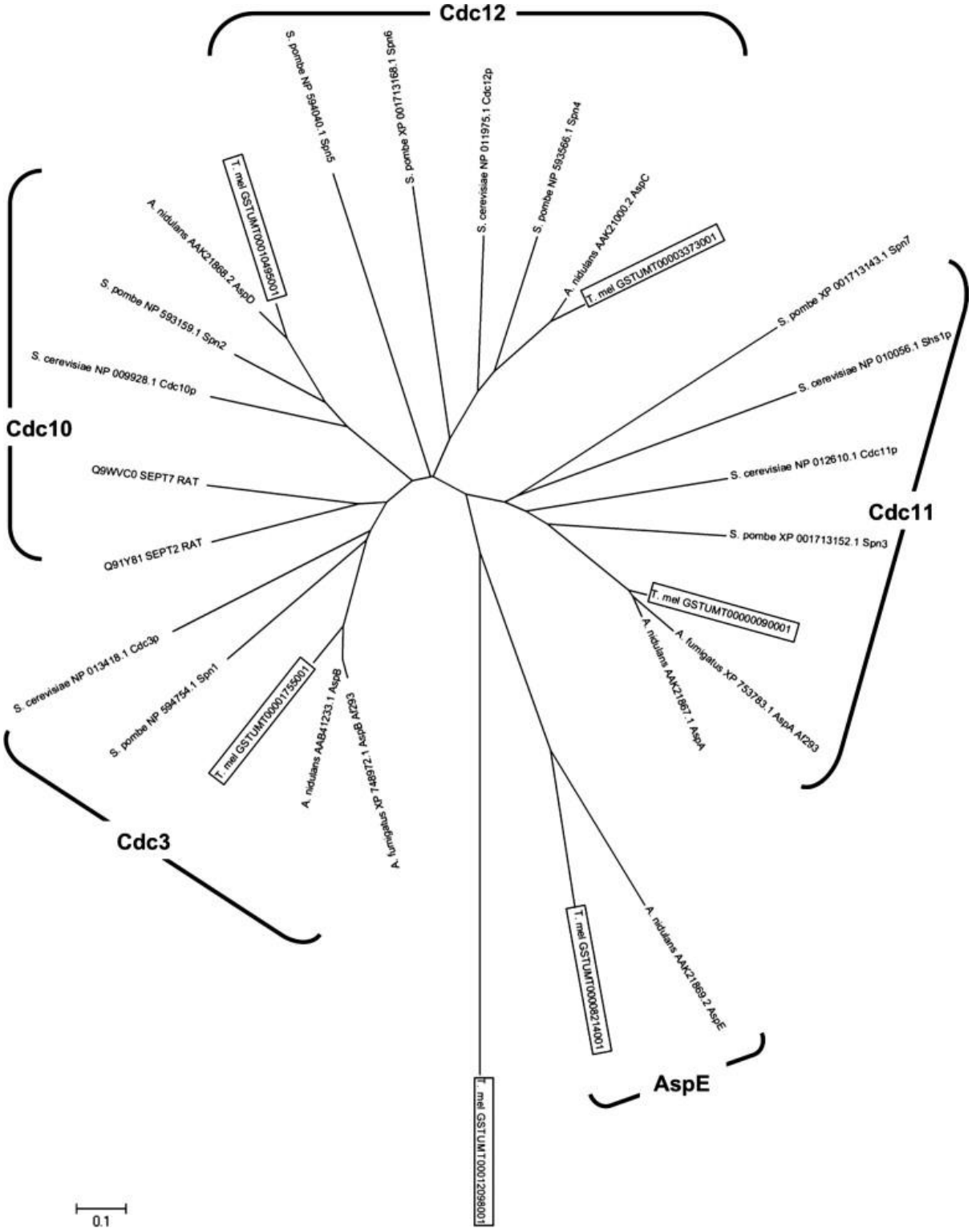


Fig. 2. Phylogenetic relationships between the yeasts (*Saccharomyces cerevisiae* and *Schizosaccaromyces pombe)* and the filamentous Ascomycetes (*Aspergillus fumigatus, Aspergillus nidulans)* based on amino acid deduced sequences for the representative septin family. The sequences were aligned using Muscle (EBi-EMBL) and the unrooted tree was constructed using the neighbor-joining method. The tree resulting from a 1000× Bootstrap neighbor-joining analysis. The *T. melanosporum* sequences analyzed in this study have been highlighted.

### 3.5. Other genes

Genes coding for proteins that are potentially related to maintaining the organelle and hyphal morphology were found in *T. melanosporum* and are reported in Table 1S. TmelCOG6 is a component of the conserved oligomeric Golgi complex, which is composed of eight different subunits and is required for normal Golgi morphology and localization, while TmelDopA, a protein belonging to the Dopey family, is required for a correct cell morphology and spatio-temporal organization of the multicellular structures in the filamentous fungus *A. nidulans*. *S. cerevisiae* Dop1 is essential for viability and affects cellular morphogenesis. The BolA-like protein family consists of the morphoprotein BolA from *E. coli* and its various homologs. Over expression of this protein in *E. coli* causes a round morphology and may be involved in switching the cell between elongation and septation systems during cell division. BolA is also induced by stress during the early stages of growth and may play a general role in stress response.

### 3.6. Microscopic analyses of truffle hyphal tips

In order to understand whether the conserved molecular machinery that emerges from the genome analysis could be confirmed at the cellular level, the *T. melanosporum* mycelium was subjected to an ultrastructural analysis. Quick-frozen and freeze substituted hyphae of *T. melanosporum* showed good ultrastructural preservation when observed under an electron microscope. Near median longitudinal thin sections of hyphae revealed details of the hyphal tip organization ( Fig. 3). Vesicles of diverse sizes and with an electron-dense content were present as separate entities or with a more organized pattern (Fig. 3, arrows). A closely packed grouping of vesicles was present just beneath the apical plasma membrane and it was identified as a component of the Spitzenkörper. The vesicles were often closely associated to the apical plasma membrane, suggesting important secretion events (Fig. 3). As expected, the microtubules extended into the apical dome and were generally positioned parallel to the long axis of the cell (Fig. 3). The microtubules were present both in the periphery and central cytoplasmic regions.

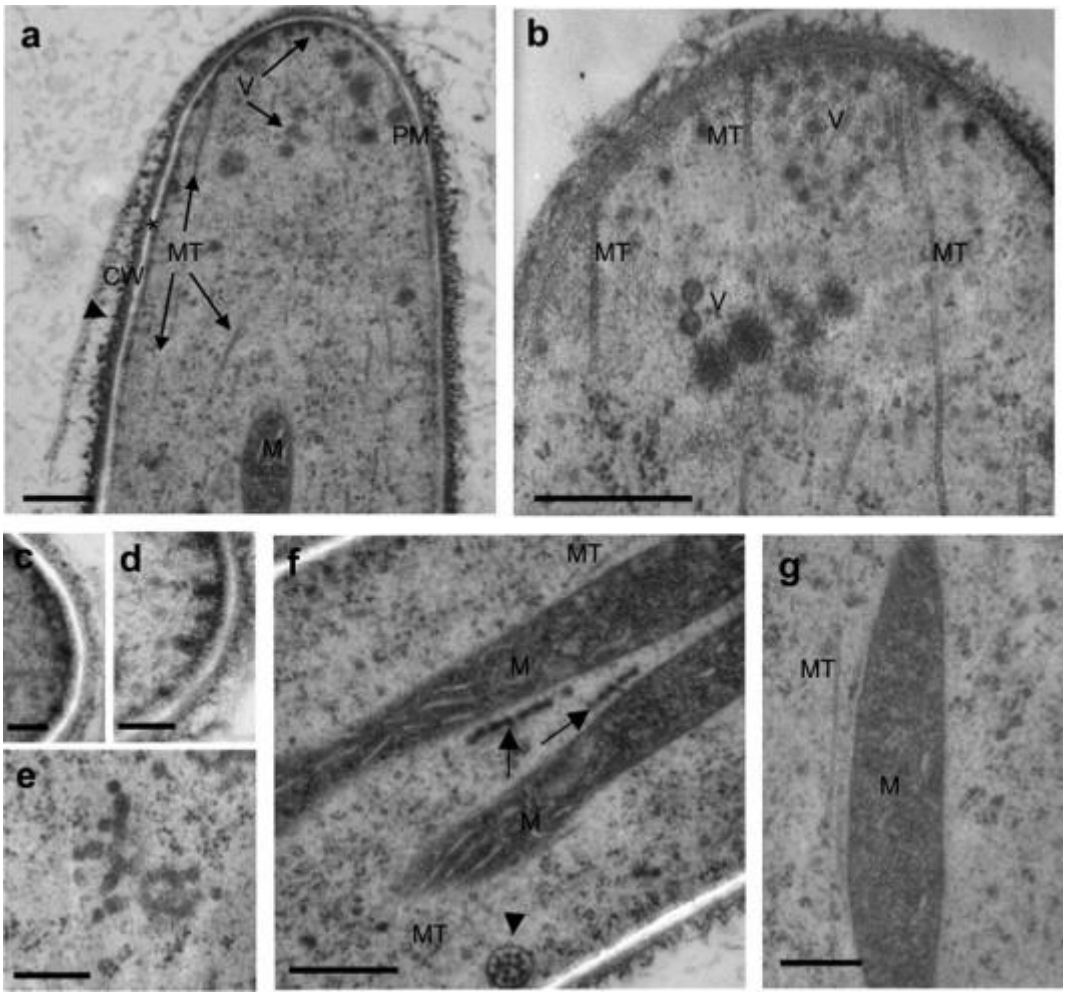


Fig. 3. Transmission electron micrographs of the hyphal tip cells of *Tuber melanosporum*. (a) Near median section through cell showing microtubules (MT), vesicles (V), mitochondrion (M), plasma membrane (PM), cell wall with transparent inner electron layer (*) and outer layer (arrowhead) with loose electron dense matrix. (b) Apical dome region of the hyphal tip. The morphological diversity of the vesicles (V) and clustering of the microvesicles and what appears to be filasomes should be noted. Microtubules (MT) are seen extending to the apical plasma membrane. (c and d) Vesicles observed in close association with the apical plasma membrane. (e) Inflated cisternae with electron dense lumen of Golgi equivalent. (f) A sub apical hyphal region with elongated mitochondria (M), multivesicolar body is also evident (arrowhead), cisterna of Golgi equivalents (arrows), and microtubules (MT). (g) Microtubules (MT) were often seen in close proximaty to mitochondria (M). Bars correspond to: 0.5 µm in figs. a–b–f–g and 0.2 µm in figs. c–d–e.

A clear ultrastructural equivalent of the Spitzenkörper as a dense, spheroidal cluster of vesicles, and cytoskeletal components as found at the tips of growing hyphae in members of the Ascomycota and Basidiomycota (Roberson et al., 2009), was not clearly observed. Spitzenkörpers are not common in other phyla and to date they have been described in only one other fungal phylum, Blastocladiomycota, and specifically in *Allomyces macrogynus* ( Vargas et al., 1993) (Fig. 3). The vesicle organization seen in Fig. 3 is similar to that characteristically seen in members of the Mucorales (Zygomycota) (Roberson et al., 2009). However, the cluster of vesicles appears to be more organized in some sections suggesting greater similarities with conventional Spitzenkörpers (Fig. 3). Further observations are required to fully clarify the tip organization for *T. melanosporum*.

Mitochondria represented the most common organelle present in the region below the tip (Fig. 3); they were sometimes associated with microtubules and inflated tubular membranous systems with electron-dense contents. These inflated membranous systems may represent portions of the Golgi apparatus, which appeared as inflated tubular and often fenestrated cisternae that varied in shape from cup-like to planar bodies. Small vesicles, probably secretory vesicles, with electron dense luminal contents, were observed in close proximity to the Golgi apparatus. Multivesicular bodies, which are also components of the endomembrane system, were present at the periphery of the hyphae.

### 3.7. Gene expression profiling

The expression of the gene encoding enzymes involved in hyphal growth was analyzed using whole-genome expression oligoarrays (Martin et al., 2010). Transcript profiling was carried out comparing free-living mycelium with ectomycorrhizal root tips and fruiting bodies. Transcripts were detected for all the 149 genes under study, indicating that all the encoding protein-genes involved in the hyphal growth machinery being considered were expressed, regardless of the fungal life cycle (Fig. 4, Table 3S).

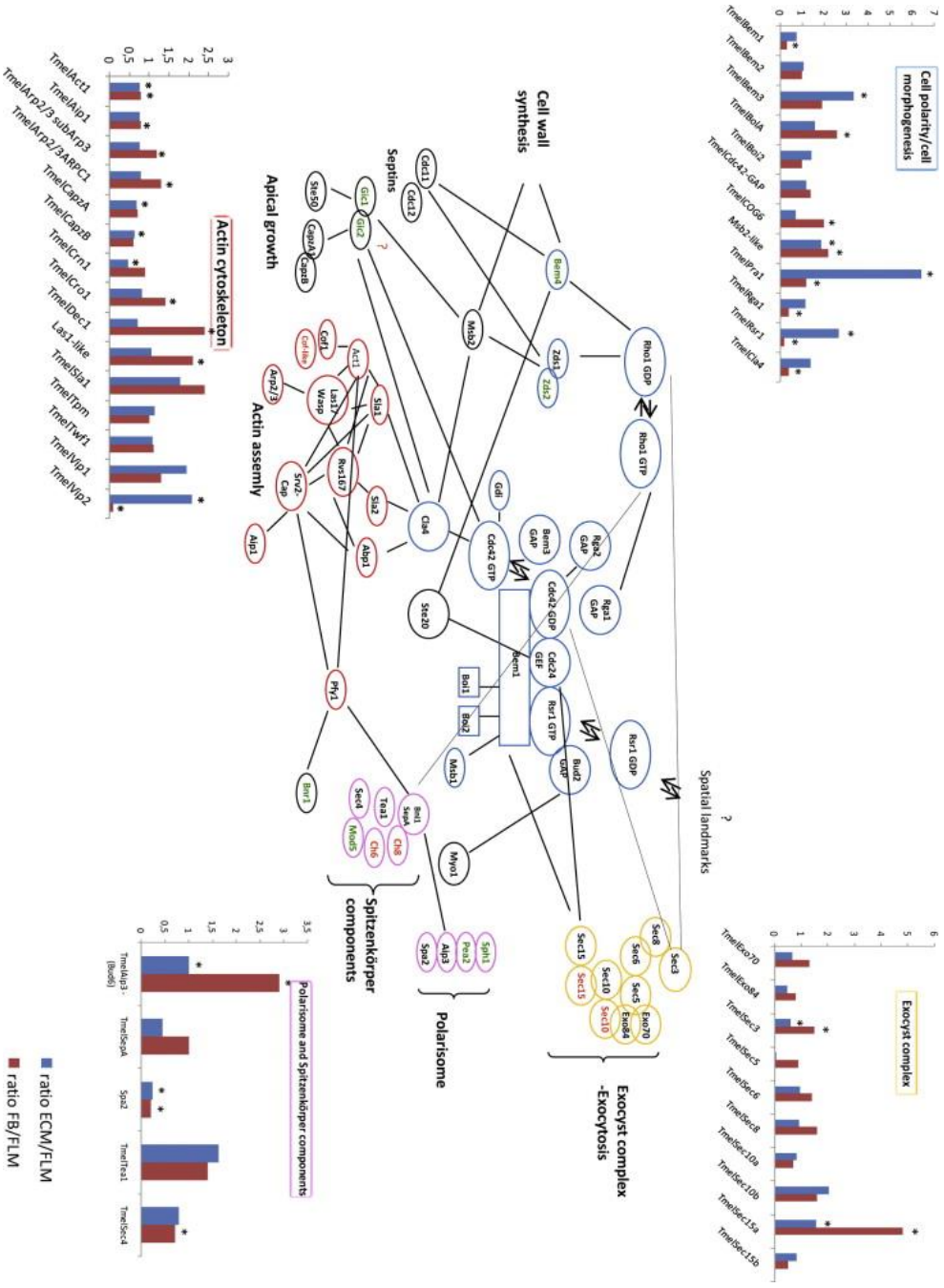


Fig. 4. Hypothesised interaction map for proteins involved in Cdc42 – regulated processes and in other development pathways in *Tuber melanosporum* compared to *Saccharomyces cerevisiae*. Only some of the annotated genes were considered in this scheme. The interaction map has been designed according to Drees et al., 2001. The proteins present in both the species are in black, the proteins present in *T. melanosporum* and absent in yeast are in red, the *Saccharomyces cerevisiae* proteins not found in *T. melanosporum* are in green. The relative expression level of several *T. melanosporum Mel28* genes involved in hyphal growth in ectomycorrhizal root tips of *T. melanosporum* (blue bars) and in fruiting bodies (red bars) are shown in the histograms. The transcription level of each gene was measured using NimbleGen whole-genome expression oligoarrays ( Martin et al., 2010) and compared to the level in free-living mycelium. $^{*}p$-value < 0.05. The genes circled in blue are part of the cell polarity/morphogenesis; the genes circled in red are involved in the actin cytoskeleton; the genes circled in pink are polarisome and Spitzenkörper components; the genes circled in yellow are exocyst complex components.

In general, only some genes showed a moderate gene expression variation during the examined three life cycle stages; the genes whose expression variation was not statistically significant are not shown in the figure.

A slight expression variation of some groups of genes coding for proteins involved in transduction signaling, linked to cell polarity and morphogenesis, was observed in the symbiotic phase: the GAPs (GTPases activating proteins) Bem 1, 2, 3, Rga1, regulators of Cdc42; Pra1, probably linked to organelle biosynthesis; the small GTPase Rsr1, upstream from the Cdc42 pathway; Cla4, an effector of Cdc42. Furthermore, important genes coding proteins involved in inositolphosphate signaling and cortical actin functioning are up-regulated in the ectomycorrhizal stage: IP6 and IP7 kinases (*TmelVip1*, *TmelVip2*) ( Mulugu et al., 2007)**.**

Interestingly, a moderate major expression of *TmelSec3* and *TmelSec15a* (twofold and fivefold respectively) – components of the exocyst complex – was observed in the fruitbody stage. Some polarisome components also showed a particular expression pattern: in particular *TmelAip3* shows a threefold higher expression in the fructification stage, while *TmelSpa2* showed higher expression in the mycelial phase ( Fig. 4, Table 3S).

A moderate up-regulation of some genes involved in the cytoskeleton structure was observed in the fructification stage (Arp complex, *TmelCro*, *TmelDec1*). Another gene that showed a discrete up-regulation in the fruitbody and mycelium stage was *TmelRmsA*, whose product is important for the hyphal growth rate ( Meyer et al., 2009; data not shown).

# 4. Discussion

Our knowledge of the molecular components involved in polarized growth in *S. cerevisiae*, *S. pombe* or *A. nidulans* and other filamentous fungi has improved rapidly over the past few years ( Banuett et al., 2008; Borkovich et al., 2004; Fischer et al., 2008; Seiler and Plamann, 2003, Steinberg, 2007a and Virag and Harris, 2006). Nevertheless, the comparison of mechanisms in different organisms shows that our understanding of the processes involved is not yet complete. Moreover, gaining a better understanding of the process in filamentous fungi would allow us to identify new proteins that determine polarized growth.

A detailed investigation of the *T. melanosporum* genome ( Martin et al., 2010) shows that 149 genes known in literature for their involvement in the hyphal growth of yeasts and filamentous or pathogenic fungi can also be identified and functionally grouped in the genome of a mycorrhizal fungus (Table 1S). The data set demonstrates the important level of conservation of these genes, through fungal genomes, regardless of their nutritional strategies.

Most of the components of the *S. cerevisiae* machinery for cell polarity and cell morphogenesis, and the cytoskeleton proved to be conserved in the truffle genome. Conversely, some proteins that are responsible for the budding yeast morphology were missing (Bem4, Bnr1, Dyn3, Gic1, Gic2, Mod5, Msb3, Msb4, Myo2, Myo3, Pea2, Rho5, Sph1, Tpm2, Zds2; Fig. 4). Hence, several aspects still need to be highlighted in order to understand which proteins play the role of the lacking ones played in yeast.

Furthermore, the comparison between the *T. melanosporum* gene set and the genome of the so far sequenced filamentous fungi, has confirmed the presence of filamentous growth-specific genes, with some exceptions (p24 dynactin, Tables 1S and 2S). In particular, cytoplasmic dynein is required in mammals for numerous intracellular transport processes; however, in *S. cerevisiae*, the

dynein function is restricted to ensuring proper nuclear movement and distribution between mother and daughter cells during cell division. Consistent with this restricted role, *S. cerevisiae* dynein and dynactin subunits are highly diverged, compared to those of higher eukaryotes, while in filamentous fungi the higher complexity of dynein complexes reflects the preponderance of filamentous growth. The lack of some components of dynein-dinactin complexes in the *T. melanosporum* genome, compared to other filamentous fungi, might suggest a link to the observed slower hyphal elongation rate in *T. melanosporum* ( Mamoun and Olivier, 1991). Two copies of the Sec10 and Sec15 genes from the exocyst complex, and a cofilin-like gene have been detected in the *T. melanosporum* genome. This enhanced complexity of exocyst elements might suggest an important exocytosis activity. The presence of two copies of these genes could be explained by a gene duplication event mediated by trasposable elements, even though no transposable elements were found for about 3000 nucleotides up or down-stream from the ORF regions. Furthermore, the gene expression analysis did not highlight a subfunctionalization for these genes/proteins in the different stages or tissues of the organism. The exocyst complex has recently been identified as an effector for five small GTPases, including Bem1, Sec4, Rho1, Rho3, Cdc42 but little is known about their role in polarized growth and cytokinesis ( TerBush et al., 1996 and France et al., 2006). For these reasons, further studies are necessary to fully understand their role in *T. melanosporum* and other filamentous fungi, and the reason behind their duplication.

The endocytosis and secretion mechanisms in the filamentous fungus *A. nidulans* can also involve a class I myosin, which is also present in *T. melanosporum* genome (TmelMyoA5) ( Pruyne and Bretscher, 2000a and Pruyne and Bretscher, 2000b). Moreover, on the basis of their similarity with yeast and several vertebrate systems (Hodge and Cope, 2000), two other *T. melanporum* myosins (TmelMyh1, class II; TmelMyo2, class V) are probably involved in cytokinesis and organelle transport, respectively. However, there is a lacking of experimental data for *T. melanosporum* and other filamentous fungi. In addition, *T. melanosporum* encodes two unconventional myosin domains linked to a chitin synthase domain (TmelChs8 and TmelChs6). These proteins have been identified in other filamentous fungi, but they are absent in yeasts, and probably have a function in cell wall synthesis and the maintenance of cell wall integrity. Myosin–chitin synthases contain an amino-terminal myosin motor-like domain and a chitin synthase domain at the carboxy terminal. The function of the motor domain is unknown, but it might involve delivering the myosin chitin synthase-attached vesicles to the growth region ( Treitschke et al., 2010). Myosin CHSs may play a role in a secretion pathway that is essential for the virulence of fungal pathogens. In *U. maydis* and *Fusarium oxysporum*, myosin CHSs are critically involved in (and required for) polarized growth during plant infection ( Weber et al., 2006 and Martín-Urdíroz et al., 2008). In *U. maydis*, it has been suggested that the Myosin Motor Domain (MMD) of ChsV supports exocytosis but not the long-range delivery of transport vesicles ( Treitschke et al., 2010).

A comparison of the *T. melanosporum* set of the master regulators of polarity Rho GTPases with their budding yeast orthologs was necessary to elucidate the similarities and differences that could contribute to the different morphologies of the two organisms. The phylogenetic analysis of *T. melanosporum* Rho proteins and the orthologous fungal sequences encoding Rho1, Rho2, Rho3, Rac and Cdc42 showed that they are evolutionarily conserved, with the exception of Rho4, which diverged with a great extent, making it difficult to predict the potential functions. Despite the high degree of similarity among the members of this family of proteins, little is known about their function in *T. melanosporum*, or in other filamentous fungi. As far as septins are concerned no significant evidence was detected; there was support for five groups of septins with orthology between kingdoms. Ascomycetes, with completed genome sequences, showed from five to eight septins while Basidiomycetes have four or five ( Pan et al., 2007). All the fungi have single Group 1 and Group 2 septins, while some fungi had multiple Group 3, Group 4 and Group 5 septins. For example, ascomycetous yeasts had three Group 3 septin paralogs. Pan et al. (2007) have reported

that *U. maydis* and *Eremothecium gossypii* are the only two filamentous fungi that are missing in the Group 5 septin.

### 4.1. Hyphal growth machinery and its transcriptional regulation

The *T. melanosporum* mycelium has symbiotic capacities, since the hyphae may establish contact with the host roots, producing a novel repertoire of morphologies, like aggregated and/or labyrinthine hyphae ( Martin et al., 2007). Comparable morphogenetic events have been described in fruitbodies where hyphae associate to produce pseudotissues that surround the reproductive structures. We wondered whether the molecular machinery that allows cell polarity and hyphal growth associated with conventional hyphal tip morphology was subjected to a differential gene expression during the life cycle transitions.

Transcriptional profiles have demonstrated a moderate but significant gene expression variation between the hyphal growth and the symbiotic structure and fruitbody formation, suggesting an involvement of the growth molecular machinery in hyphal morphogenesis during transition from the hyphal growth to the symbiotic (mantle and Hartig net) and reproductive structures (spores, fruiting bodies).

In particular, an up-regulation has been observed in the symbiotic stage of *TmelRsr1*, a small GTPase that interprets the spatial landmark (axial or bipolar) and recruits another GTPase module that consists of the Rho GTPase Cdc42, its guanine nucleotide exchange factor (GEF) Cdc24, and its GTPase-activating proteins (GAPs) Bem3 and Rga1/2 ( Casamayor and Snyder, 2002, Park and Bi, 2007, Pruyne and Bretscher, 2000a and Pruyne and Bretscher, 2000b). This module in turn recruits the actin cytoskeleton, which polarizes secretion towards the site of growth.

AgRsr1 (Bud1 in yeast) is required in *A. gossypii* for actin organization, for normal hyphal growth and branching, and for positioning of the polarisome component AgSpa2 ( Bauer et al., 2004 and Knechtle et al., 2003) which interacts with numerous proteins, such as those involved in the protein kinase pathway, actin-interacting proteins and with the other polarisome components ( Sheu et al., 1998, Shih et al., 2005 and Virag and Harris, 2006b). The Agrsr1 null mutant is characterized by false branch initiation, pausing and re initiation of growth which results in bulges along the hypha, and the transient appearance and disappearance of AgSpa2, which correlates with phases of pausing and growth (Bauer et al., 2004). In *C. albicans*, another fungus closely related to *S. cerevisiae*, CaRsr1/Bud1 defects result in random budding in yeast cells and in defects in germ tube emergence ( Yaar et al., 1997).

The activation of the pathway involved in hyphal branching is in agreement with the increased branching observed during ectomycorrhizae development (as shown in *T. borchii* by Menotta et al., 2004). Experimental interaction studies will be conducted in order to understand the activation pathway responsible for branching.

Another gene that shows an interesting marked major expression (sevenfold) during the symbiotic phase is *TmelPra1*. This encodes a prenylated Rab acceptor that exhibits a Gdi displacement factor (GDF) activity for endosomal Rab–Gdi complexes *in vitro* ( Martincic et al., 1997). Rab GTPases are proteins localized to organelles of the secretory and endocytic pathways (Lazar et al., 1997). They are involved in organelle biogenesis and the maintenance of membrane-bound organelles and play a role in the regulation of intra organelle trafficking and intra cellular organelle transport. It has been reported that Pra1 protein has a large COOH-terminal hydrophobic region, and it binds multiple Rabs (Calero et al., 2002) and that catalyzes the dissociation of endosomal Rab–Gdi complexes in *S. cerevisiae* ( Sivars et al., 2003). In *S. cerevisiae*, the overexpression of Yip3p

(Pra1p) inhibits cellular growth, and it can play a crucial role in vesicles trafficking ( Geng et al., 2005).

The high expression of *TmelPra1* in the ectomycorrhizal stage can lead to the activation of Rab proteins, inducing several mechanisms that are guided by the Rab proteins involved in organelle biogenesis and vesicle intracellular trafficking.

It has been reported (Meyer et al., 2009 and Meyer et al., 2010) that very early events that precede hyphal branching indicate the interruption of hyphal elongation, and set the stage for the subsequent formation of two new centers of polarization growth. Hence, the Spitzenkorper detaches from the apex and disintegrates. Subsequently, an actin re-polarization and increased cell wall biosynthesis occur. The necessity of establishing two new vesicles centers during branch formation may explain the high degree of expression of *TmelPra1* in the symbiotic phase.

Furthermore, the apical branching event is accompanied by the synthesis of phospholipid signaling molecules (P and IP, in that genes that encode the correspondent enzymes) in *A. niger* ( Meyer et al., 2009). This evidence is in agreement with the higher expression of IP6 and IP7 kinases (*TmelVip1*, *TmelVip2*) in the ectomycorrhizal stage in *T. melanosporum*. The inositol pyrophosphate (PP-IP) produced by Vip1 in fact represents a high-energy signaling molecule that is involved in such diverse processes as vacuole biogenesis, stress response, DNA repair, cell wall synthesis, telomere maintenance, and phosphate homeostasis.

The gene expression analyses highlighted an interesting increased expression of some genes during the fruitbody stage. Since the hyphae in the fruitbodies associate to produce pseudotissues that surround the reproductive structures, an intense hyphal growth rate and secretive activity is expected.

The genes that show a higher activity, more or less marked, at this stage are involved in the hyphal growth elongation rate and in the secretory processes aimed at the deposition of material to the apex. Among them *TmelCog6* encodes a component of the Golgi complex, that is involved in the refining of neosynthesized proteins designated to the secretion. Analogously, the increased secretion in the fructification stage is reflected in an increased expression of some components of the exocyst complex, in particular *TmelSec15a*, which is involved in secretive processes. In *A. nidulans*, SecC, a homolog of *S. cerevisiae* Sec3, accumulates at the plasma membrane anterior to the Spitzenkörper and may function as a landmark for secretion ( Taheri-Talesh et al., 2008). Moreover, a higher expression of *TmelAip3*, a polarisome component and of *TmelBoi1A* ( Kasai et al., 2004 and Knechtle et al., 2006) can reflect a higher rate of hyphal growth.

Interestingly, *TmelRmsA* is more expressed in the fructification and in the vegetative stage. This gene (ortholog of *Avo1p*/*Sin1*) encodes for a protein that is a component of a kinase complex, named Torc2, which is conserved throughout the eukaryotic evolution and which mediates spatial control of cell growth by regulation of the actin cytoskeleton ( Wang and Roberts, 2005). This complex has been shown to be essential for the determination of cell polarity in *S. cerevisiae*, *Dictyostelium discoideum* and mammalian cells ( Jacinto and Lorberg, 2008). In *S. pombe*, it interacts with the mitogen-activated protein (MAP) kinase Sty1 ( Wilkinson et al., 1999) and induces pseudohyphal growth in stress conditions. It has recently been shown that it is also present and functional in the filamentous fungus *A. niger* ( Meyer et al., 2009 and Meyer et al., 2010). In *A. niger*, it has been shown that a single point mutation in the *RmsA* gene is responsible for defective hyphal growth, suggesting its involvement in the elongation rate of the parent hypha prior to the appearance of apical branches ( Reynaga-Peña and Bartnicki-Garcia, 2005 and Meyer et al., 2009). The *RmsA* gene is also present in the *T. melanosporum* genome and the alignment with the ortholog

proteins of yeast and *A. niger* has allowed us to confirm the presence of the conserved domains. A higher gene expression of *TmelRmsA* in the fruitbody stage might reflect that a higher elongation rate is required to envelop the reproductive structures.

Despite the presence of almost all the genes involved in filamentous hyphal growth, a clear ultrastructural equivalent of the Spitzenkörper, which is expected in Asco- and Basidiomycetes (Harris et al., 2005 and Roberson et al., 2009), has yet to be identified within the *T. melanosporum* hyphal tip. We predict that the slower hyphal extension rates that are characteristic of *T. melanosporum* lead to a small number of secretory vesicles associated with the Spitzenkörper and that these vesicles are not as well organized as they are in other faster growing hyphal species.

The molecular and cellular analyses have raised many questions regarding fungal morphogenesis: as a perspective, it would be interesting to gain a better understanding of the role of the identified hyphal growth genes in plant/fungal interaction context.

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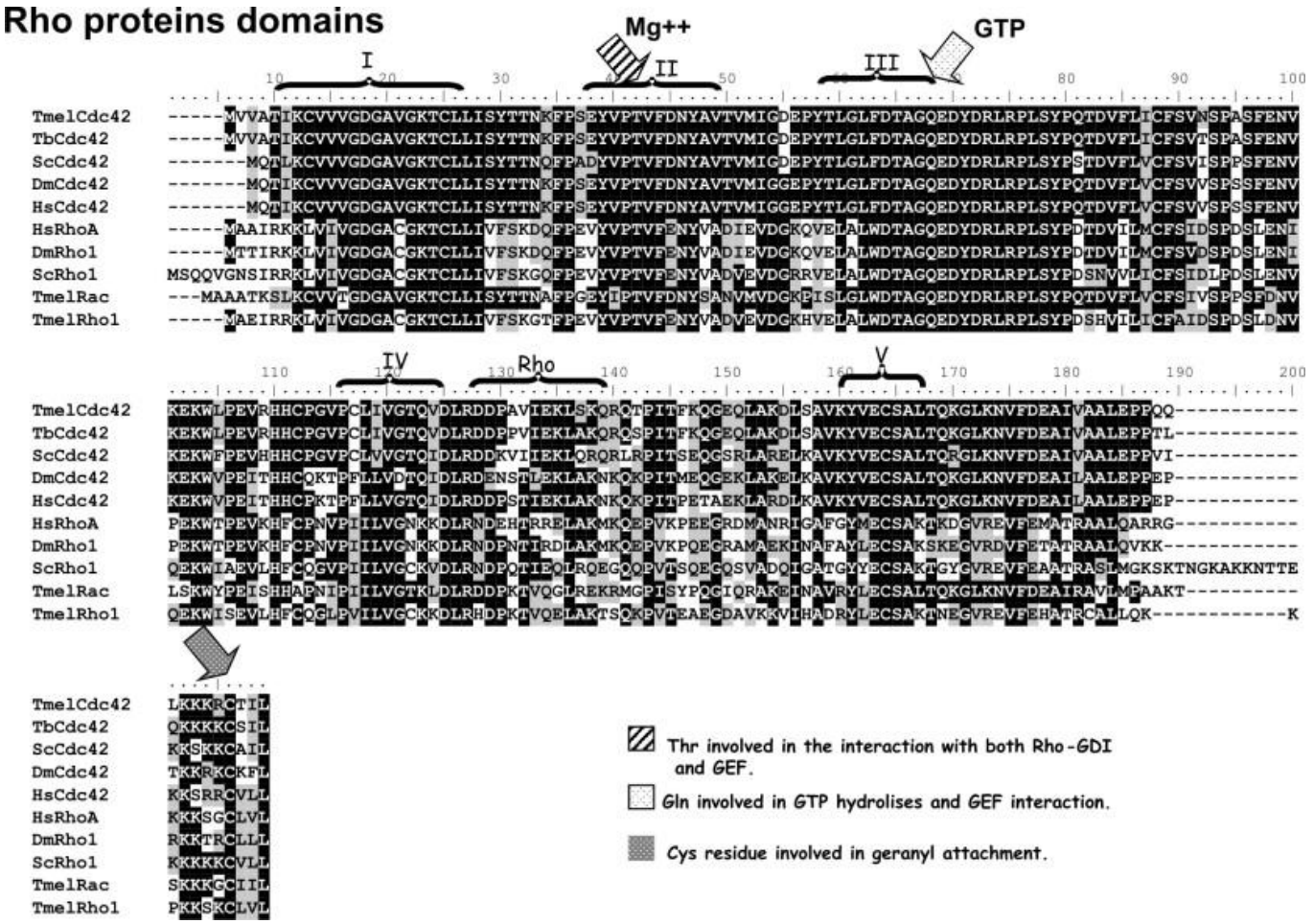
# Appendix A. Supplementary material




Supplementary Figure 1. Figure 1S: Multiple alignment of Rho proteins from *T. melanosporum* and representative species. Sequences were aligned with ClustalX and the output file was subsequently edited manually. The dashes indicate gaps introduced for optimal alignment purposes. Residues identical or similar in at least four sequences are boxed in black or grey, respectively. Secondary structure elements are indicated above the aligned sequences.

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