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Sugar transporters in the black truffle *Tuber melanosporum*: from gene prediction to functional characterization

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ABSTRACT

In a natural forest ecosystem, ectomycorrhiza formation is a way for soil fungi to obtain carbohydrates from their host plants. However, our knowledge of sugar transporters in ectomycorrhizal ascomycetous fungi is limited. To bridge this gap we used data obtained from the sequenced genome of the ectomycorrhizal fungus *Tuber melanosporum* Vittad. to search for sugar transporters. Twenty-three potential hexose transporters were found, and three of them (*Tmelhxt1*, *Tmel2281* and *Tmel131*), differentially expressed during the fungus life cycle, were investigated. The heterologous expression of *Tmelhxt1* and *Tmel2281* in an *hxt-null Saccharomyces cerevisiae* strain restores the growth in glucose and fructose. The functional characterization and expression profiles of *Tmelhxt1* and *Tmel2281* in the symbiotic phase suggest that they are high affinity hexose transporters at the plant–fungus interface. On the contrary, *Tmel131* is preferentially expressed in the fruiting body and its inability to restore the *S. cerevisiae* mutant strain growth led us to hypothesize that it could be involved in the transport of alternative carbon sources important for a hypothetical saprophytic strategy for the complete maturation of the carpophore.

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1. Introduction

Sugar transport in cells is a vital function for all organisms. It is particularly important for the ectomycorrhizal (ECM) symbiosis, a mutualistic plant–fungus association essential to the persistence of both tree and fungal mycelium, mainly in nutrient-poor soils. ECMs represent one way that trees and fungi in forest ecosystems can overcome carbohydrate limitations (Nehls et al., 2007). In natural forest soils, ECM fungi help their hosts by supplying required minerals and water, since mycelium can proliferate extensively within soils (Högberg and Högberg, 2002) and efficiently prospect for nutrient resources, often at considerable distances from the plant roots. Furthermore, fungi protect their host plants against stresses such as drought or soil pollution (Domínguez Nuñez, 2008, 2009). The plant in turn redirects its photosynthates toward mycorrhized roots and exchanges them with its fungal partner. In this mutually beneficial partnership, fungi have direct and privileged access to simple carbohydrates, which are extremely scarce in forest soils (Nehls, 2008). Hence, ECM fungi must possess an efficient hexose transporter systems.

Tuber melanosporum Vittad. is an ECM ascomycetous filamentous fungus that associates with the fine roots of trees such as oaks (*Quercus* spp.) and hazelnuts (*Corylus avellana*). After the establishment of ectomycorrhizas, the fungal life cycle is completed with the production of hypogeous fruiting bodies, called truffles, which have considerable economic importance due to their organoleptic properties (Trappe and Claridge, 2010).

The eukaryotic fungal system in which sugar transport has been the most thoroughly studied is the yeast *Saccharomyces cerevisiae* (Boles and Hollenberg, 1997). In this organism, 18 genes, HXT1–HXT17 and GAL2, encode proteins belonging to the monosaccharide transporter family, whereas two other proteins, called Snf3 and Rgt2, act as glucose sensors and control the expression of the hexose transporters according to the availability and concentration of carbon sources (Lagunas, 1993; Özcan and Johnston, 1999).

Conversely, little is known about sugar transport in mycorrhizal symbiosis. Regarding arbuscular mycorrhizal (AM) fungi, few transporters have been characterized: a monosaccharide transporter GpMST1 from *Geosiphon pyriformis*, which forms a symbiotic

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relationship with the cyanobacterium *Nostoc punctiforme* (Schüßler et al., 2006), and the high-affinity monosaccharide transporter MST2 from *Glomus–Medicago truncatula* symbiosis (Helber et al., 2011).

Concerning ECM fungi, the first transporters were characterized from *Amanita muscaria*, namely *AmMst1* and *AmMst2* (Nehls, 2004; Nehls et al., 1998). *AmMst1*, constitutively expressed in fungal hyphae under all growth conditions, shows an increased expression at high levels of monosaccharides and has a stronger preference for glucose than for fructose uptake (Nehls et al., 1998); the second identified potential sugar transporter, *AmMst2*, has not yet been investigated at the functional level. A predicted fungal hexose transporter has been shown to be up-regulated during the entire course of ECM development between *Betula pendula* (birch) and basidiomycete *Paxillus involutus* (Breakspear and Momany, 2007), but this gene has not been characterized. Among *Tuber* species, *Tbhxt1* from *Tuber borchii* is the first high affinity glucose transporter that has been identified. Considering the expression pattern of the *Tbhxt1* gene and the kinetic characteristics of the encoded protein, it is mainly responsible for carbohydrate uptake by soil-growing hyphae and the reduction of sugar leakage from hyphae and is not associated with the symbiotic process (Polidori et al., 2007). The large gap in our knowledge regarding hexose import into ECM was bridged by López et al. (2008), who identified 15 potential sugar transporter proteins in the genome of the basidiomycete ectomycorrhizal fungus *Laccaria bicolor*. Among the 15 putative transporters, four genes were confirmed as functioning by heterologous expression in yeast and six genes were active in ECM root tips, suggesting their role in carbon assimilation. On the basis of gene expression patterns and import kinetics, the authors postulated two potential functions for these transporters: the uptake of carbohydrates by soil-growing hyphae and the uptake of hexoses at the plant–fungus interface.

The availability of the complete genome sequence for the ECM fungus *T. melanosporum* (Martin et al., 2010) provides the opportunity to screen for genes encoding proteins of the Major Facilitator Superfamily (MFS). Among 91 MFS transporter members previously identified by Martin et al. (2010), in this paper, 23 genes predicted to encode transporters of sugar were identified and functional characterization of three selected members were described.

2. Materials and methods

2.1. Biological materials and growth conditions

T. melanosporum mycelia were grown in modified Woody Plant Medium, pH 6.5–6.7, as described in Iotti et al. (2005). Fruiting bodies were collected in Central Italy and characterized by morphological and molecular methods based on carpophore and spore shape as well as multiple polymerase chain reaction (PCR) amplification, respectively. *T. melanosporum–Quercus robur* ectomycorrhizas were obtained as previously described (Giomaro et al., 2005).

For functional studies *Saccharomyces cerevisiae* mutant strains EBYVW.4000 (*MATa Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3 leu2-3, 112 ura3-52 trp1-289 his3-Δ1 MAL2-8^c SUC2*) (Wieczorke et al., 1999) and BY4742 (*stl1*) (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YDR56w::kanMX4*) were used. The strain EBYVW.4000 was maintained as described in Boles and Hollenberg (1997) and the strain BY4742 was grown as described in Kayingo et al. (2009).

2.2. In silico genome automatic annotation and manual curation

First, we searched in the Tuber DB Tuber genome database (http://mycor.nancy.inra.fr/IMGC/TuberGenome/) using as a hit the PF00083 signature, which corresponds to the sugar transporter family (Sugar_tr) in the MFS superfamily (Pfam clan CL0015). The gene models (putative genes) identified were used for additional searches in the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN and BLASTP algorithms. All the gene models selected were inspected manually and modified when necessary 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). Subsequently, in order to improve the sequences, each curated orthologous 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: *Neurospora crassa*, *Magnaporthae grisea*, *Botrytis cinerea*, *Aspergillus nidulans* and *S. cerevisiae*. Finally, the gene models identified were classified into two sub-families based on discriminating signature diagnostics for sugar transporters (IPR005828, IPR003663) using the InterPro database (http://www.ebi.ac.uk/interpro/) (Table 1). The sequences were further analyzed using fungal sugar proteins with proven hexose import capability as a template (Boles and Hollenberg, 1997; Helber et al., 2011; López et al., 2008; Nehls et al., 1998; Polidori et al., 2007; Wieczorke et al., 1999). For the three GSTUMTs characterized in this investigation, the BLAST search was also extended to the Ascomycota genomes available at the fungal genomic portal MycoCosm (http://jgi.doe.gov/fungi) (Grigoriev et al., 2014).

2.3. Phylogenetic analysis

Predicted protein sequences from the present genome survey were used for extensive database searches for both homolog sequences and sequences that are closely related phylogenetically. The sequences were aligned using the CLUSTALX package (Thompson et al., 1997). Phylogenetic analyses were performed with the PHYLIP software package, version 3.63 (Felsenstein, 2004), and inferred using neighbor joining, maximum parsimony and maximum likelihood. All methods retrieved a similar tree topology. Bootstrap analyses were based on 200 re-samplings of the sequence. The MEGA 6.06 program was used to plot the tree files (Tamura et al., 2013).

2.4. Sugar transporter isolation

Putative sugar transporters were cloned by PCR performed on a *T. melanosporum* cDNA plasmidic library pYES-DEST52 and using specific primer pairs described in Table S1, Box 1. PCRs were performed in a final volume of 25 μl containing 1× reaction buffer, 200 μM dNTPs, 200 pmol of each primer pair and 0.5 U of TITANIUM™ Taq DNA Polymerase (Clontech). The mixture was incubated for 1 min at 95 °C and then subjected to 30 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at the specific temperature for each primer pair and 1.45 min of extension at 68 °C. Amplicons of the expected size (~1500 bp) were ligated using T4 DNA Ligase (Promega) into the pGEM-T vector (Promega). *Escherichia coli* XL1 Blue cells were transformed with each construct, and the recombinant clones were identified. Plasmid DNAs were isolated using the QIAGEN Plasmid MiniKit and sequence-verified.

2.5. Heterologous complementation in the yeast mutant strain EBYVW.4000

For heterologous expression in the *S. cerevisiae* mutant strain EBYVW.4000, the cDNAs of each sugar transporter were obtained using the specific primer pairs reported in Table S1, Box 2

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