# Sugar Uptake and Channeling into Trehalose Metabolism in Poplar Ectomycorrhizae.

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Dedicated to Alejandra and Arturo. “Thanks for being my permanent supply of peace and love”, and also to my beloved parents for their unconditional support.

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1 Summary ... 1
2 Zusammenfassung ... 2
3 Introduction ... 4
3.1 Soil and rhizosphere ... 4
3.2 Mycorrhiza ... 4
3.2.1 Types of mycorrhizae ... 5
3.2.2 Ectomycorrhizae ... 6
3.3 Ectomycorrhizal model organisms ... 8
3.4 Monosaccharide uptake and metabolism in ectomycorrhizal fungi. ... 10
3.5 Trehalose: properties and occurance. ... 12
3.5.1 Trehalose metabolism ... 12
3.5.2 Functions of Trehalose ... 13
4 Goal of the Thesis ... 15
5 Results and Discussion ... 16
5.1 The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil-growing hyphae ... 16
5.1.1 Phylogenetic analysis of the *Laccaria bicolor* sugar porter gene family ... 16
5.1.2 Impact of carbohydrate nutrition on sugar transporter gene expression ... 17
5.1.3 Impact of nitrogen nutrition on sugar transporter gene expression ... 19
5.1.4 The sugar porter gene family of *Laccaria bicolor*: function in fruit bodies . 21
5.1.5 Functional analysis of selected sugar porters ... 23
5.1.6 Summary of The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil-growing hyphae ... 24
5.2 Increased trehalose biosynthesis in Hartig net hyphae of ectomycorrhizas ... 25
5.2.1 Isolation of Hartig net and sheath hyphae from established ectomycorrhizas 25
5.2.2 cDNA and deduced protein sequences of *trehalose biosynthesis genes* ... 26
5.2.3 *AmTPS*, *AmTPP* and *AmTP* expression is strongly increased in hyphae of the Hartig net ... 27
5.2.4 Impact of sugar and nitrogen supply on *AmTPS*,*AmTPP* and *AmTP* expression ... 29

5.2.5 Expression analysis of genes involved in trehalose metabolism in *A. muscaria* fruiting bodies. .......... 29
5.2.6 Summary of Increased trehalose biosynthesis in Hartig net hyphae of ectomycorrhizas .......... 33
6 Author´s contribution .......... 34
7 References .......... 35
8 Curriculum Vitae .......... 46
9 Appendix .......... 47

# 1 Summary

Ectomycorrhizal associations are characterized by the bidirectional exchange of nutrients and carbohydrates.The plant rewards the fungal partner a continues support of easily degradable carbohydrates that can make up to 30% of the total carbon fixed by the plant host. To understand how a strong fungal carbon sink is generated, the carbon partitioning in the poplar (*Populus tremula x tremuloides) –Amanita muscaria* ectomycorrhizal symbiosis was studied. Two aspects were followed: monosaccharide uptake and carbon storage.

The creation of a strong carbohydrate sink by the fungus is related to the efficiency of fungal hexose uptake at the plant-fungus interface. In the genome of the currently sequenced model fungus *Laccaria bicolor* all putative sugar porter genes were identified and their transcript abundance was determined in symbiotic (*Populus tremula x tremuloides*/*Laccaria bicolor)* and non-mycorrhizal mycelia. Gene expression profiles were separated according to carbon availability and ectomycorrhiza formation. The functional characterization of selected proteins was carried out by heterologous expression in a yeast mutant containing no intrinsic hexose uptake systems.

A quick conversion of hexoses taken up by fungal hyphae is a further prerequisite for the formation of a strong fungal carbohydrate sink Trehalose is supposed to be an intermediate store as well as a transport metabolite of fungal carbon and is present in large quantities in *A. muscaria*. Using RT-PCR, the transcript levels of genes coding for enzymes involved in the biosynthesis of trehalose, such as trehalose-6-phosphate synthase (TPS), trehalose-6-phosphate phosphatase (TPP) and trehalose phosphorylase (TP), were determined. All three genes were upregulated in hyphae located at the plant-fungus interface, indicating an increased fungal trehalose biosynthesis capacity in symbiosis. Furthermore, the upregulation of these genes was correlated with an increased TPS protein activity and elevated trehalose content at the symbiotic interface. Since the expression of trehalose biosynthetic genes was not under metabolic control they are presumably developmentally regulated.

Finally, trehalose biosynthesis genes displayed a dynamic expression pattern in developing fruiting bodies, supporting the idea that there is a high trehalose demand during fruiting body development that cannot be supported only by the extradical mycelium.

## 2 Zusammenfassung

Ektomykorrhizale Beziehungen zeichnen sich durch den bidirektionalen Austausch von Nährstoffen und Kohlenhydraten aus. Die Pflanze belohnt den Pilzpartner mit einer andauernden Versorgung mit leicht zersetzbaren Kohlenhydraten, die bis zu 30% des durch den Pflanzenwirt fixierten Gesamtkohlenstoffs ausmachen. Um zu verstehen, wie eine starke Kohlenstoffsenke generiert wird, wurde die Kohlenstoffaufteilung in der Ektomykorrhizasymbiose der Pappel *Populus tremula x tremuloides* mit *Amanita muscaria* untersucht. Zwei Aspekte wurden verfolgt: Aufnahme von Monosacchariden und Kohlenstoffspeicherung.

Die Erzeugung einer starken Kohlenstoffsenke hängt mit der Effizienz der Hexoseaufnahme durch den Pilz an der Pflanze-Pilz-Schnittstelle ab. Im Genom des sequenzierten Modellpilzes *Laccaria bicolor* wurden alle vermuteten Zuckertransportergene identifiziert und die Häufigkeit ihrer Transkripte in symbiotischen (*Populus tremula x tremuloides / Laccaria bicolor*) und nicht-mykorrhizalen Myzelien bestimmt. Anhand ihrer Expressionsprofile wurden die Gene gemäß der Kohlenstoffverfügbarkeit und Ektomykorrhizaformation aufgeteilt. Die funktionelle Charakterisierung von ausgewählten Proteinen wurde durch heterologe Expression in einer Hefemutante durchgeführt, die keine eigenen Hexoseaufnahmesysteme besitzt.

Eine schnelle Umwandlung von durch Pilzhyphen aufgenommenen Hexosen ist eine weitere Voraussetzung für die Bildung einer starken Kohlenstoffsenke. Trehalose dient dem Pilz sowohl als Zwischenspeicher als auch als Transportmetabolit für Kohlenstoff und findet sich in grossen Mengen in *A. muscaria*. Mittels RT-PCR wurden die Transkriptmengen von Genen, die an der Biosynthese von Trehalose beteiligt sind, wie z.B. Trehalose-6-Phospat-Synthase (TPS), Trehalose-6-Phosphat-Phosphatase (TPP) und Trehalose-Phosphorylase, bestimmt. Alle drei Gene wurden in Hyphen an der Pilz-Pflanzen-Schnittstelle verstärkt exprimiert, was auf die gesteigerte Trehalose-Biosynthese-Kapazität des Pilzes in der Symbiose hinweist. Außerdem korellierte die verstärkte Expression dieser Gene mit einer gesteigerten TPS-Proteinaktivität und mit einem erhöhten Trehalosegehalt an der symbiotischen Schnittstelle. Da die Expression von biosynthetischen Genen der Trehalose nicht unter metabolischer Kontrolle steht, sind sie vermutlich durch die Entwicklung reguliert.

Abschliessend zeigten die Trehalosebiosynthesegene ein dynamisches Expressionsmuster in sich entwickelnden Fruchtkörpern, was die Hypothese unterstützt, dass während der Fruchtkörperentwicklung ein großer Trehalosebedarf besteht, der nicht allein durch das extraradicale Myzel gedeckt werden kann.

# 3 Introduction

## 3.1 Soil and rhizosphere

Soil structure has a strong impact on plant yield. Aggregates determine the mechanical and physical properties of a soil such as retention and movement of water, aeration, and temperature and are thus an important factor controlling germination and root growth (Younes et al., 2000).

Plant roots but also microorganisms contribute to soil organic mater by synthesis, accumulation, and secretion of a diverse array of compounds (referred as root exudates) and thereby modulate soil aggregate stability. These compounds serve also an important role as attractants and repellants in the rhizosphere, the narrow zone of soil immediately surrounding the root system (Travis et al., 2003; Bais et al., 2001; Estabrook and Yoder., 1998). Root exudates have traditionally been grouped into low- and high-$M$r compounds. High-$M$r exudates primarily include mucilage (high-$M$r polysaccharides) and proteins and are important for soil aggregate stability. Low-$M$r compounds such as amino acids, organic acids, sugars, phenolics, and various other secondary metabolites are believed to comprise the majority of root exudates and act as messengers that stimulate biological and physical interactions between roots and soil organisms (Steinkellner et al., 2007).

## 3.2 Mycorrhiza

The term mycorrhiza**,** which literally means *fungus-root*, was first applied by the German forest pathologist Frank in 1885, to describe the association between fungal mycelium and tree roots. Since then, we have learned that the vast majority of land plants form symbiotic associations with fungi. As estimated, 95% of all plant species belong to genera that characteristically form mycorrhizae. This mutual interaction is thus believed to have played an important role in the successful colonization of the land by plants (Brundrett, 2002; Wang, 2006).

The major function of mycorrhizae is a better carbohydrate nutrition of the fungal partner as well as mycorrhizal fungi convey a range of additional benefits to their host plant including increased resistance to foliar-feeding insects (Gange and west.1994) and soilborne pathogens (Whipps,2004), as well as tolerance to salinity (Feng et al., 2002) and heavy metals (Diaz et al.,1996).
There are some generalizations that can be made, concerning mycorrhizae:
1) Mycorrhiza infection takes place only on the smallest order of roots. These are the root tips that are still growing. 2) In all mycorrhizae only rhizodermal and cortical cells of the root are in contact with the fungus. 3) The mycorrhizal association is typical for nearly all plant families with the exception of the *Brassicaceae*, *Chenopodiaceae*, *Cyperaceae*; and aquatic plants. 4) It is believed that mycorrhizae forming plants would loose a substantial part of their competitive capacity in their natural habitats without this symbiotic relationship.

### 3.2.1 Types of mycorrhizae

Based on morphology and the plants species that are involved several different types of mycorrhizae are recognized. They can be grouped into three major categories:
**Ectomycorrhizae** are frequently characterized by an external sheath of mycelium that is formed around the fine root. Fungal hyphae lyse only the middle lamella but do not penetrate secondary cell walls and thus invade only the extracellular space between cortical cells (Hartig Net; details see below).
**Endomycorrhizae** are characterized by a lack of an external fungal sheath, the penetration of secondary cell walls of cortical cells by the mycelium and virtual fungal growth within plant cells.

The most common type of endomycorrhizae is the Arbscular Mycorrhiza (AM) which occurs in more than 80% of plant species. The partners of the symbiosis comprise plants without real roots, such as pteridophytes or gametophytes of some mosses (lycopods), as well as angiosperms and gymnosperms (Smith et al., 1997). The AM fungi are obligate symbionts as they cannot persist without establishing symbiosis with a host plant. No known sexual stage is reported for AM fungi (George et al., 1995).
Ather types with endomycorrhizal habitat are ericoid and orchid mycorrhizas. The term ericoid is applied du to the fact that this mycorrhizal association is found exclusively with

plants in the order of *Ericales*, while orchid mycorrhizae are unique in the life cycle of orchids.

**Ectendomycorrhizae** are an anatomical intermediate between ectomycorrhizae and endomycorrhizae. A fungal sheath around the infected root is frequently found but reduced in thickness and structuring and hyphal cells do penetrate the secondary cell wall of cortical cells as endomycorrhizal fungi.

In all types of mycorrhizae, fungal hyphae explore a larger soil and litter volume and thus strongly increase the absorptive surface of the host plant.

### 3.2.2 Ectomycorrhizae

Ectomycorrhizal (ECM) fungi include at least 6000 species, primarily of basidiomycotic and, to a lower content, ascomycotic and zygomycotic background (Brundrett, 2002). They are typicaly associated with temperate woody plants (*Pinaceae, Fagaceae, Betulaceae, Myrtaceae*), but also with some monocotyledons and ferns (Wilcox, 1996). In the northern hemisphere, ectomycorrhizal fungi appear to be primarily associated with forest trees while in the tropics, only a few forest trees form ectomycorrhizas. However, also in the southern hemisphere a broad range of trees, shrubs and herbs is capable of ectomycorrhiza formation.

Ectomycorrhizal (ECMs) associations are frequently found in soils that are poor in available nutrients. Here, litter accumulation occurs due to (temperature or humidity based) seasonality and a thus limited bacterial degradation activity, which is taken over in part by fungal mycelia. Soil-growing hyphae explore the litter for nutrients and can constitute a large part of the ectomycorrhizal fungal colony. When mycorrhizal fungal hyphae recognize an emerging fine root of a compatible plant partner, they direct their growth towards it (Martin et al., 2001) and colonize the root surface forming a sheath or mantle of hyphae, which encloses the root and isolates it from the surrounding soil (Blasius et al., 1986). After or parallel to the sheath formation, fungal hyphae grow inside the infected fine root, forming highly branched structures in the apoplast of the rhizodermis (angiosperms) and in the root cortex (gymnosperms). This so-called Hartig net generates a large surface of area of interaction between both partners (Kottke and Oberwinkler, 1987). Hyphal aggregates and cords or rhizomorphs interconnect different

parts of a colony (such as mycorrhiza and fruiting body; Cairney et al., 1991), enabling the exchange of nutrients and carbohydrates over long distances.
Both hyphal networks of ectomycorrhizas have diverse functions (Harley and McCready, 1952; Harley and Smith, 1983; Kottke and Oberwinkler, 1987; Smith and Read, 1997). The Hartig net serves as an interface between plant and fungus and is adapted to the exchange of plant-derived carbohydrates for fungus-derived nutrients. The function of the fungal sheath, in contrast, is that of an intermediate storage for a) nutrients that are delivered by soil growing hyphae and are intended for delivery to the Hartig net, and b) carbohydrates that are taken up by the hyphae of the Hartig net and are going to be transported towards the soil-growing mycelium.

An essential part of ectomycorrhizal fungal colonies are macroscopic sporocarps (Luoma et al., 2004). The different tissues and cell types of fruiting bodies are the result of a tightly regulated differentiation process. As an example, the development of *Agaricus bisporus* fruiting body starts with hyphae consolidating into a mycelial cord by adhesive mucilaginous substances (Umar & Van Griensven, 1998). Multiple primordia, consisting of frequently branching and actively growing hyphae, develop within or in close relation to these mycelial cordsprimordial and grow within 2 days to a length of about 6 mm. Their development either ceases or continues through a so-called histo-organogenetic stage (Umar & Van Griensven, 1997). At this stage, vertically oriented hyphae, which form the future stipe, and a dense mass of radial oriented hyphae, which form the future cap, can already be distinguished microscopically (Craig et al., 1977; Craig et al., 1979; Umar & Van Griensven, 1997).

The fruit bodies of basidiomycetes are the most complex differentiated structures formed by fungal mycelia. Fruit bodies are composed of three main regions, the cap, gills or tubular structures and the stipe. The cap protects the extensive gill/tubular surface basidiospores are generated, while the stipe serves to raise these regions into a position suitable for spore dispersion.
Fruiting body formation is highly influenced by the physiological conditions and the nutritional state of the mycelium (Flegg and Wood 1985) and depends, for instance, on a strictly balanced availability of carbon and nitrogen (Moore, 1998; Kües und Liu 2000 ). Not surprisingly, the presence of a symbiontic plant may allow successful fruiting body formation for ectomycorrhizal fungi (Danell and Camacho, 1997).

### 3.3 Ectomycorrhizal model organisms

Among the most studied ectomycorrhizal associations today are those of *Populus tremula x tremuloides* with *Amanita muscaria* or *Laccaria bicolor*.
*Populus* is a genus of trees which includes the cottonwoods, poplars, and aspens. About 30 poplar species are divided into six sections (*Populus*, Aegiros, Tacamahaca, Leucoides, Turanga, Abaso) comprising about 10% of the family members of the *Salicaceae* (Boes et al., 1994).
The *Populus* section contains *Populus tremula* (Common Aspen, Trembling Aspen or Eurasian Aspen, Europe, northern Asia) and *Populus tremuloides* (Quaking Aspen or Trembling Aspen, North America), whereas *Populus trichocarpa* is found in the Tacamahaca section (Black Cottonwood; also known as Western Balsam Poplar or California Poplar).

Poplar was chosen as the first tree model to be sequenced because of its relatively compact genome (Brunner et al., 2004) which is about three times larger than *Arabidopsis* but 50 times smaller than that of pine (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Several expressed sequence tag (EST) projects exist, and whole genome expression analysis is feasible. Furthermore, polar can be easily grown under laboratory and greenhouse conditions and its physiology can be manipulated due to a well developed transformation system.

The cosmopolitan symbiotic fungus *Amanita muscaria* (Fig. 1; the fly agaric) is a member of the familiy *Amanitaceae*, order *Agaricales* (gilled mushrooms). This family contains several species that are valued for edibility and flavor but many others are deadly poisonous. Many Amanita species are known to form ectomycorrhizas (ECM) with arboreal trees such as Birch, Pine or Fir, in both Europe and America.

Different EST- and transcriptome databases are available and a genome project is currently under progress.

**Figure 1**: *Amanita muscaria* fruit body.
(The picture was taken from en.xihalife.com)

The second model fungus *Laccaria bicolor* (Fig.2; common name: bicoloured deceiver) is a member of the *Tricholomataceae*, a large order of ectomycorrhizal and saprobic basidiomycetes. Like *A. muscaria*, *Laccaria* is a cosmopolitan and a common genus of Agaricales which has been reported from numerous ectomycorrhizal plant communities. The physiological ecology of L. bicolor is well studied among ectomycorrhizal taxa, because it grows rapidly in pure culture and its mycorrhizae are easily established with tree roots under laboratory conditions. Finally, this species is used in large-scale commercial inoculation programs in forest nurseries worldwide to enhance growth of tree seedlings.

**Figure 2**: *Laccaria bicolor* fruit body.
(The picture was taken from: bioimages.org.uk)

The complete *Laccaria* genome sequence was published in 2008 by an international consortium (Martin et al., 2008) and arrays for genome-wide expression analysis are available. The fungus genome contains about 20,000 genes, twice as many as *Neurospora crassa* or *Phanerochaete chrysosporium*. Bioinformatic analysis of the genome revealed unexpected aspects of *Laccaria* biology such as the presence of genes potentially associated with wood decay and indicated a general tendency of higher basidiomycotic fungi for expanding gene families. Especially G-protein-coupled receptors, virulence-associated genes and enzymes involved in secondary metabolism revealed 30 to 50 % more members as other sequenced fungal genomes. Furthermore, sequencing of about 40,000 ESTs from various cDNA libraries have shown that alternatively spliced transcripts are abundant. The genome is rich in transposons belonging to various class I and II families.

### 3.4 Monosaccharide uptake and metabolism in ectomycorrhizal fungi.

Due to the low content of readily useable carbohydrates in the soil, ectomycorrhizal fungi are ecologically dependent on a continuous carbon support by the plant during the growth season (Harley and Smith, 1983; Smith and Read, 1997; Leake et al., 2001). In ectomycorrhizal symbiosis up to 30% of total plant photoassimilates can be transferred to the fungus to enable its proliferation (Finlay and Söderström 1992; Söderström 1992). The driving force for carbon allocation in vascular plants is the consumption at the sink site. Mycorrhizas attract carbohydrates much more efficiently than non-mycorrhizal fine roots (Bevege et al., 1975; Cairney et al., 1989), indicating a strong sink created by fungal hyphae in symbiosis.

It is assumed that sucrose, the major long-distance transported carbohydrate of most plants, is exported into the apoplast and hydrolyzed by a plant-derived acid invertase (Lewis and Harley, 1965b; Salzer and Hager, 1991; Hampp and Schaeffer, 1999; Nehls et al., 2001a). The resulting hexoses are then taken up by fungal cells as well as by plant root cells (Lewis and Harley, 1965a; Palmer and Hacskaylo, 1970; Chen and Hampp, 1993; Nehls et al., 2000). Thus, a competition for hexose uptake by plant cells might control the carbohydrate drain. A prerequisite for rapid uptake of monosaccharides is an efficient membrane transport system. In the baker yeast *Saccharomyces cervisiae*,

more than 20 functional sugar transporters have been identified (Boles and Hollenberg, 1997). However, only a small number of ectomycorrhizal hexose transporter genes have yet been characterized, i.e. two hexose transporter genes for *Amanita muscaria* (AmMst1, Nehls et al., 1998; AmMst2, Nehls, 2004) and one for *Tuber borchii* (Polidori et al., 2007). Based upon limited gene expression data and protein function it has been supposed that the fungal hexose uptake capacity is strongly increased in Hartig net hyphae (Nehls et al., 1998; Wiese et al., 2000; Nehls et al., 2001a; Nehls, 2004).

According to NMR (Martin et al., 1988; Martin et al., 1994) and biochemical assays (Hampp et al., 1995; Schaeffer et al., 1996; Kowallik et al., 1998) imported sugars are used in large amounts for the generation of ATP, carbon skeletons for biosynthetic purpose, and carbohydrate storage (Fig. 3).

Ectomycorrhizal fungi produce a series of fungus-specific sugars and sugar alcohols when there is no limitation in carbon supply (Martin et al., 1985; Martin et al., 1987; Martin et al., 1988; Martin et al., 1998). Dependent on the fungus, different pools of storage carbohydrates can be distinguished: polyols (mannitol, arabitol, erythritol; data not shown), oligosaccharides (trehalose; Schaeffer et al., 1995; Wallenda, 1996; Hampp and Schaeffer, 1999), and the long chain carbohydrate glycogen.

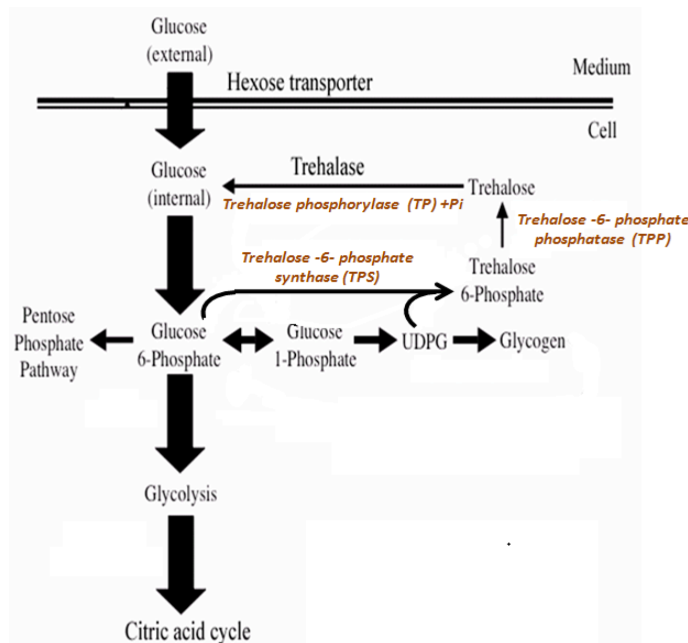



**Figure 3:** Metabolic Pathways of internalized glucose in ectomycorrhizal fungal hyphae.

Glucose, taken up by hexose importers is phosphorylated by hexokinase/glucokinase and can be directed into three different pathways: glycolysis, trehalose biosynthesis, glycogen formation**.**

### 3.5 Trehalose: properties and occurance.

Trehalose (1-α-glucopyranosyl-1-α-glucopyranoside) is a non reducing, particularly stable disaccharide formed by two glucose moieties (Schiraldi, et,al., 2002).

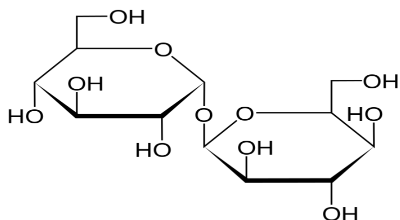



**Figure 4**: Structure of α,α1,1-trehalose

Although there are three possible anomers of trehalose, i.e., αβ-1, 1-,β,β-1,1-, and α,α1,1-, only the α,α-trehalose has been isolated from living organisms. Trehalose is found in all kingdoms; bacteria, yeast, fungi, plants, and invertebrates (Elbein et,al., 2003; Elbein, 1968), suggesting an important role in the biological world (Koen et al., 2000).

Trehalose is quite common in yeast and fungi where it occurs in spores, fruiting bodies and vegetative cells (Nwaka and Holzer, 1998). In addition many species of lichens (Linderberg, 1955) and algae (Augier, 1954) contain this disaccharide, although in considerably lower concentrations than found in yeast. Trehalose is probably also present in many higher plants, since it has been isolated from the resurrection plant (*Selaginella lepidophylla*) (Adams et al.,1990) but also from *Arabidopsis thaliana* (Müller et al., 1990).

#### 3.5.1 Trehalose metabolism

Different pathways for the biological synthesis of trehalose have been described (Avonce et al., 2006; Elbein et al., 2003). The most widely reported and best studied occurs by means of two enzymatic pathways (Avonce, 2006). The first pathway, discovered about 50 years ago, is present in eubacteria, archaea, fungi, insects, and plants. It involves two irreversible enzymatic steps catalyzed by *trehalose-6-phosphate synthase (TPS)* and *trehalose- phosphatase (TPP). TPS* catalyzes the transfer of glucose from UDP-glucose to glucose 6-phosphate forming trehalose 6-phosphate (T6P)

and UDP. *TPP* than dephosphorylates T6P to trehalose and inorganic phosphate (De Smet et al., 2000; Elbein et al., 2003). In the second pathway, trehalose phosphorylase (TP) catalyses the hydrolysis of trehalose into glucose-1-phosphate and glucose. As this reaction is thermodynamicaly balanced and thus reversible, trehalose phosphorylase catalyses also the phosphorolysis of trehalose in the presence of inorganic phosphate (Fig. 3). As the equilibrium constant is around 7 at cytosolic pH (pH 6.5) the reaction is supposed to be thermodynamically directed towards trehalose synthesis and not its degradation (Parrou et al., 2005; Eis et al., 1998). Trehalose phosphorylase has been isolated from a limited number of fungi, including *Pichia fermentans* (Schick et al., 1995), and the basidiomycetes *Grifola frondosa*, *Schizophillum commune*, *Flammulina velutipes* (Eis et al., 1998; kitamoto et al., 1988) and the mushroom *Agaricus bisporus* (Wannet et al., 1998). In L. bicolor S238N – H82, a single gene encoding TP is present in the genome (Fajardo et al., 2007).

The irreversible hydrolysis of trehalose into two molecules of glucose is catalyzed by trehalases. Most fungi possess two types of trehalose hydrolases, referred as “acid” and “neutral” trehalases in accordance with their pH optimum.

### 3.5.2 Functions of Trehalose

Trehalose is undoubtedly one of the key players in the protection of living organisms against different environmental stresses (Sussich et al., 2001). “Resurrection plants,” nematodes, brine shrimps, and yeast can survive under extreme desiccation, where up to 99% of the cellular water can be lost. Trehalose probably interacts directly with the dry protein by hydrogen bonding between its hydroxyl groups and polar residues in the protein of membranes (Carpenter and Crowe, 1989). To enable these protective functions trehalose content can reach levels up to 10–20% of the dry weight under desiccation.

High levels of trehalose also prevent cells against oxygen radicals that cause amino acid damage in proteins. In baker yeast, trehalose-defective mutants showed a much higher content of damaged protein after a brief exposure to oxygen stress, indicating trehalose acting as a free radical scavenger (Elbein et al., 2003).

Trehalose levels may vary greatly in cells depending on their growth stage, their nutritional stage, and other environmental conditions prevailing at the time of measurement (Elbein et al., 2003). In fungi, trehalose is an important component in spores, where trehalose hydrolysis is found to be a major event during early germination, suggesting that this sugar is a carbon store for production of energy and carbon skeletons (Thevelein, 1984; d'Enfert et al., 1999). Some reports have shown that ascospores of *Neurospora tetrasperma* contain as much as 10% trehalose (Sussman, 1959).

In contrast to many ascomycetes certain basidiomycotic ectomycorrhizal fungi, accumulate trehalose also in vegetative hyphae when growing on glucose as sole carbon source. Furthermore, trehalose accumulation has been observed upon ectomycorrhiza formation (Martin et al., 1988; Ineichen & Wiemken, 1992; Wallenda, 1996; Rangel-Castro et al., 2002). Therefore, trehalose has been supposed to function as carbohydrate store under these conditions. Especially at the symbiotic interface trehalose biosynthesis can be essential for the generation of a strong carbohydrate sink as imported glucose has to be quickly converted into fungal metabolites (Lunn et al., 2006). Furthermore, in analogy to sucrose in plants trehalose is also considered as a transport sugar in ectomycorrhizal fungal colonies (Söderström et al., 1988).

# 4 Goal of the Thesis

The infection of plant fine roots by ectomycorrhizal fungi strongly increase the proportion of plant carbohydrates that is directed towards the root system. It is therefore supposed that ECM fungi are capable in: a) an efficient carbohydrate uptake from the common interface and b) a fast conversion of plant-derived carbohydrates into fungal compounds. Monosaccharides are thought to be a major fungal carbon source in symbiosis. The goal of this thesis was thus to investigate the fungal monosaccharide uptake in symbiosis and the role of trehalose in the conversion of monosaccharides into fungal storage compounds.

Due to their specific properties, two fungal models (*Amanita muscaria* and *Laccaria* bicolor) have been used for these investigations together with *Populus tremula x tremuloides* as host plant. As ectomycorrhizas are composed of two different fungal networks with distinct physiology, the advantage of *A. muscaria* to isolate both networks separately from each other were used for selected aspects. In particular, we were interested in A) the generation of a Petri dish based system to enable controlled conditions. B) To distinguish the nutritional and developmental signals that could control fungal gene expression of mycelia grown in submerse cultures under defined nutritional conditions. C) To perform functional enzyme assays in order to link protein function and transcriptional control.

# 5 Results and Discussion

## 5.1 The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil-growing hyphae

Although ectomycorrhizal (EM) fungi are facultative saprotrophs, the analysis of the *Laccaria bicolor* genome revealed that this ectomycorrhizal basidiomycete is poorly adapted to degradation of carbon-rich lignocellulose (Deveau et al., 2008). Hence, the formation of mycorrhizal symbiosis is of pivotal importance to face fungal carbohydrate limitation in forest soils. Here, EM fungi have direct and privileged access to root exudates because: a) the root surface is covered by a sheath of hyphae and fine roots are thus isolated from the surrounding soil and b) fungal hyphae grow inside the infected fine root, forming highly branched structures in the apoplast, the so-called Hartig net (e.g. Blasius et al., 1986), to enable nutrient and metabolite exchange with the host. Constant import of plant-derived carbohydrates via the symbiotic interface plays a crucial role for the creation of a strong carbohydrate sink by the fungus.

The major source of carbon used by most ectomycorrhizal hyphae comes from the sucrose provided by the host plant (Nehls et al., 2007). The lack of invertase in *L. bicolor* (Martin et al., 2008), confirms that sucrose is converted into fructose and glucose into the symbiotic apoplastic space by the plant invertase. Therefore, one focus of this work was on the identification and functional characterization of fungal hexose importers.

### 5.1.1 Phylogenetic analysis of the *Laccaria bicolor* sugar porter gene family

By searching the JGI website (available at http://genome.jgi-psf.org/ Lacbi1/Lacbi1.home.html) for annotated putative hexose transporter genes and further screening of the genome sequence by using BlastN and tBlastN and fungal sugar importers with proven hexose import capability (Doehlemann et al., 2005; Polidori et al., 2007; Wieczorke et al., 1999; Nehls et al., 1998) as a template, we coulde identify a total of 15 potential sugar-transporter genes. Similar gene numbers as in *Laccaria bicolor* were found in the genomes of *Coprinopsis cinerea* (18; reachable in http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html), *Phanerochaete chrysosporium* (16; Martinez et al., 2004), *Ustilago maydis* (19; Kämper

et al., 2006), *Aspergillus niger* (20; Pel et al., 2007) and *Saccharomyces cerevisiae* (20; Boles & Hollenberg, 1997). A significantly larger number was found only in *Cryptococcus neoformans* (Loftus et al., 2005), with 48 potential sugar transporter genes.

For phylogenetic analysis (based on deduced protein sequences), gene models of all identified *L. bicolor* hexose transporters, were manually inspected and the best fitting proteins were used. From six genes (Lacbi1:304755, Lacbi1:313180, Lacbi1:301992, Lacbi1:385212, Lacbi1:380081, Lacbi1:183424) the corresponding cDNAs were amplified for functional analysis. For one of these genes (Lacbi1:301992) the cDNA sequence differed from the best predicted model and the corrected protein sequence (Accession No. AM998533) was used for phylogenetic analysis. Ten out of the 15 *L. bicolor* identified putative sugar porter proteins fell into three different clusters supported by bootstrap values above 60 %. The four deduced *Laccaria bicolor* proteins of cluster 1 cluster together with 19 out of 20 *Saccharomyce*s members of the SP gene family and both ectomycorrhizal fungal sugar transporters that were functionally characterized so far (TBHXT1 from *Tuber borchii*, Polidori et al., 2007 and AmMST1 from *Amanita muscaria*, Nehls et al., 1998). With the exception of members of this protein cluster, only two further fungal proteins of the sugar porter gene family were so far successfully functionaly characterized. STL1 from *Saccharomyce cerevisiae* (which is closely related to Lacbi1:191542), a member of cluster II, was shown to be a glycerol transporter (Ferreira et al., 2005), while BcFRT1 from *Botrytis cinerea,* which is a member of cluster III and closely related to Lacbi1:385212 was characterized as a fructose importer (Doehlemann et al., 2005).

### 5.1.2 Impact of carbohydrate nutrition on sugar transporter gene expression

Since in *A. muscaria* sugar porter gene expression is influenced by sugar supply (Nehls et al., 2001a; Nehls et al., 2007; Nehls et al., 1998) this effect was also investigated in *L. bicolor*. When mycelia were grown in liquid culture without a carbon source, a conspicuous enhanced expression was observed for five transporter genes. This indicates a strong demand of fungal hyphae for sugar uptake capacity under carbon limitation/starvation, which is in agreement with the observation of Polidori et al. (2007) in *Tuber borchii*.

The evaluated genes can be grouped according to their maximum level of expression in

substrate mycelium as follows: one to 10 mRNA molecules per 10 000 rRNAs (eight genes: *Lacbi1:380081* > *Lacbi1:183424*> *Lacbi1:304755*, *Lacbi1:142821* and *Lacbi1:385212* >*Lacbi1:305352*, *Lacbi1:301992*, *Lacbi1:191542*), 0.1 to 0.9 mRNA molecules per 10 000 rRNAs (two genes: *Lacbi1:313180* > *Lacbi1:298959*), and transcript abundances below 0.1 mRNA molecules per 10 000 rRNAs (four genes: *Lacbi1:297020* > *Lacbi1:314210* > *Lacbi1:300971* > *Lacbi1:397934*). After sugar addition to carbon starved mycelia, three different patterns in gene expression could be distinguished (Fig.5). No correlation between the expression levels or patterns and the phylogenetic relationship of the respective proteins was observed.

A) Four genes revealed an unchanged transcript levels or some fluctuations unrelated to fungal growth.

B) Three genes revealed a temporally restricted induction of gene expression.

C) Seven genes showed an either fast or slow down regulation of their transcript levels.

The complete set of data is found in the attached paper (Fajardo et al., 2008).

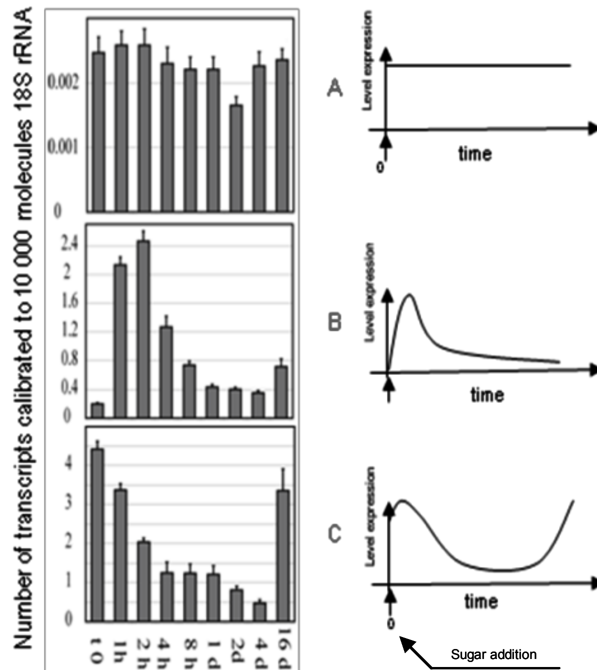



**Figure 5**: Patterns of sugar porter gene expression after sugar addition to carbon starved L. bicolor mycelia. *L. bicolor* mycelia were pre-cultivated in liquid MMN (Hampp et al., 1996) in absence of any carbon source for one week. After medium exchange and addition of glucose (final concentration 10 mM), mycelia were further cultivated for up to 16 days (without a change of the respective growth medium) and mycelial sample were taken at different time. Total RNA was isolated and first-strand cDNA was synthesized after DNA removal. Expression analysis was performed by quantitative RT-PCR using gene specific primers and was calibrated to 10,000 molecules of 18S rRNA.removal**.**

When compared to carbohydrate starvation, a clear reduction of gene transcripts was observed for most (10 out of 14) of the potential sugar transporters in the presence of glucose or fructose. The transcript levels of these genes were reduced in the presence of either glucose or fructose. With the sole exceptions of *Lacbi 1: 397934*, high glucose concentration (10mM) strongly reduced gene expression, while the impact of fructose was weaker. The presence of sucrose or raffinose had only a minor impact on the transcript abundance for the majority of the genes.

These results are in clear contrast to results obtained from *A. muscaria*, where both investigated sugar porter genes were clearly up-regulated upon glucose addition to carbon starved mycelia. A further distinct difference between *A. muscaria* and *L. bicolor* was the behavior of fungal mycelia on glucose uptake after monosaccharide addition to carbon starved hyphae. While no lag phase for glucose uptake was observed for *L. bicolor* (this work), *A. muscaria* hyphae (Wiese et al., 2000), but also those of *Hebeloma cylindrosporum* (Salzer & Hager, 1991) needed about one day before glucose import was maximal. Together these data clearly indicate substantial differences of ECM fungi with respect to sugar-dependent regulation of monosaccharide transporter gene expression.

### 5.1.3 Impact of nitrogen nutrition on sugar transporter gene expression

Fungal carbohydrate and nitrogen nutrition are interconnected and thus may affect each other at the regulatory level (Baars et al., 1995; Nehls, 2004). Therefore, the impact of nitrogen nutrition on *L. bicolor* SP gene expression was investigated. Fungal mycelia were grown in liquid culture in the presence of four different nitrogen sources which are found in forest soils (nitrate, ammonium, urea, and amino acids, as described by Nehls et al., 1999) or in the absence of a nitrogen source (Fig.6). Eight out of 14 putative sugar porter genes showed no or just minor changes in their transcript levels while four genes revealed a tendency towards a mild gene repression in the presence of any nitrogen source. Nevertheless, together these data clearly indicated that nitrogen nutrition had only minor effects on the expression of SP genes.

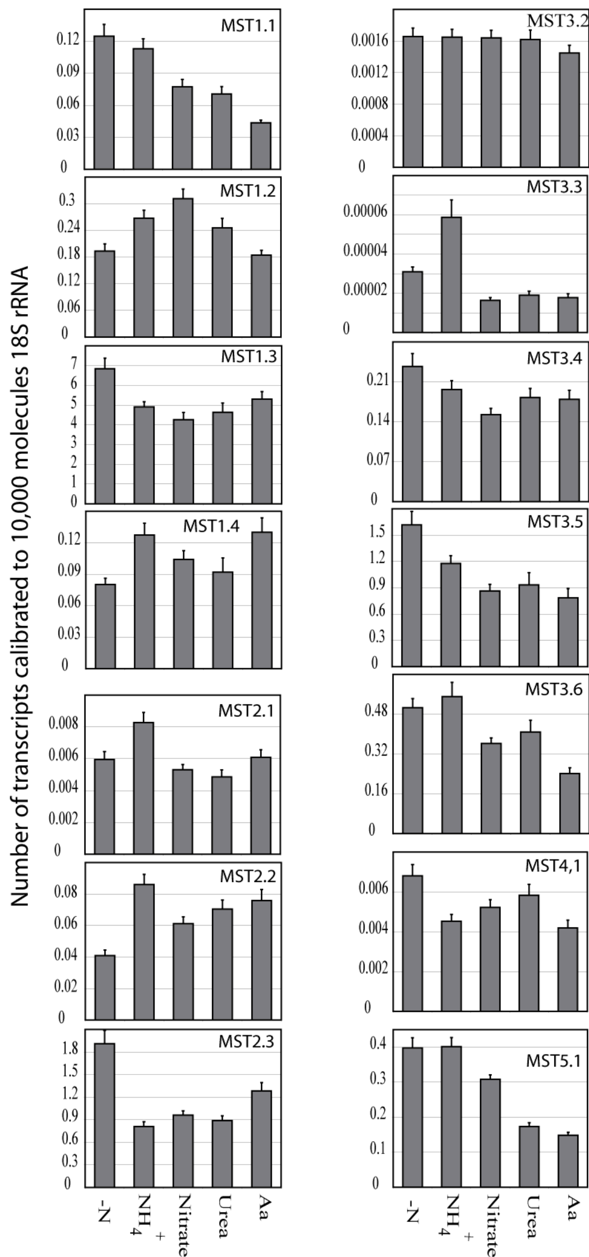



**Figure 6**: Impact of nitrogen nutrition on gene expression of members of the sugar porter gene family in *Laccaria bicolor*.

Fungal mycelia of Laccaria bicolor were pre-cultivated in liquid MMN (Hampp et al., 1996) in absence of any nitrogen source (-N) for one week before addition of different nitrogen sources (final concentration of 2 mM). Samples were collected after 2 days of incubation (with a change of the respective growth medium twice a day). Total RNA was isolated from mycelial samples. Expression analysis was performed by quantitative RT-PCR using gene specific primers and was calibrated to 10,000 molecules of 18S rRNA. Aa, amino acids (Nehls et al., 1999).

### 5.1.4 The sugar porter gene family of *Laccaria bicolor*: function in fruit bodies

Fungal fruiting bodies dramatically increase in size during their development. The main reason for this is fast cell elongation and not cell division. As they do contain large amounts of sugars, it has been supposed that sugars (mainly glucose and trehalose) may function as osmolytes for cellular water import. Due to the high turgor of the respective cells and the large hexose content, glucose leakage might be a serious problem that might be solved by increased sugar porter gene expression. Therefore, the role of hexose importers in the formation of the fruiting bodies was evaluated in *Laccaria bicolor*.

When compared with the extraradical mycelium (Erm), ten out of a total of 15 genes (*Lacbi1: 301992, Lacbi1: 380081, Lacbi1: 191542, Lacbi1: 300971, Lacbi1: 297020, Lacbi1: 142821, Lacbi1: 298959, Lacbi1: 314210, Lacbi1: 183424, Lacbi1: 385212*) showed elevated transcript levels upon formation of fruiting bodies (Fig.7). The expression of two genes (Lacbil:300971 and Lacbil:142821) was up regulated mainly in the stipe of fruiting bodies while others were similar high expressed in mycorrhizas and fruiting bodies (*Lacbil:301992, Lacbil:297020*, *Lacbil:385212*). *Lacbil:397934* revealed strong gene expression only in the extraradical mycelium and other sugar porter genes showed a complex pattern of their transcript levels, which cannot be easily explained by a developmental or nutritional regulation.

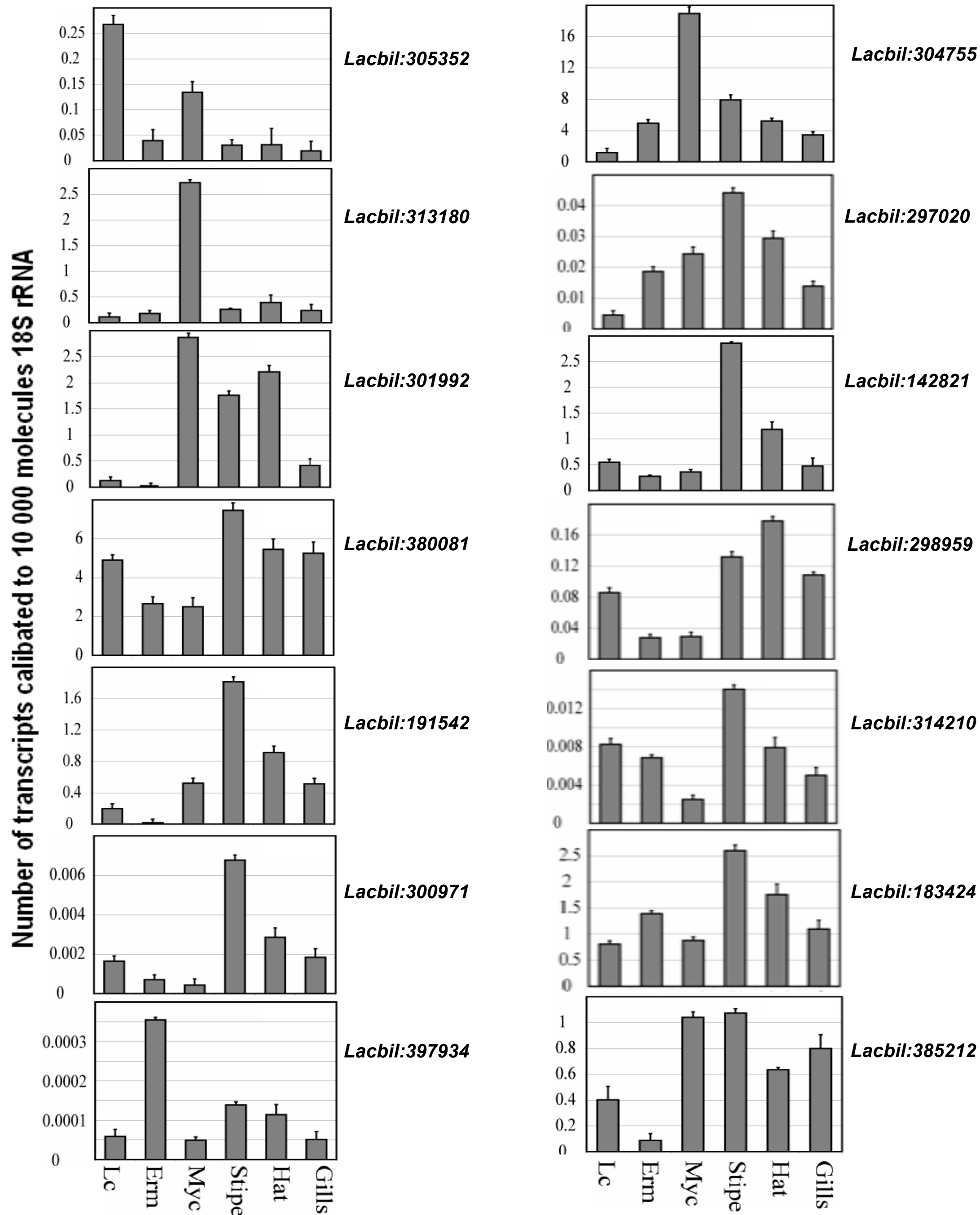



**Figure 7**: Gene expression of members of the sugar porter gene family upon fruiting body formation.

Total RNA was isolated from *L. bicolor* mycelia grown in liquid culture with 10 mM glucose as carbon source for two days prior to harvest (Lc), the extraradical mycelium (Erm), ectomycorrhizas (Myc) and fruiting bodies that were dissected into the stipe, gills and remaining hat structures. Expression analysis was performed by quantitative RT-PCR using gene specific primers and was calibrated to 10,000 molecules of 18S rRNA.

### 5.1.5 Functional analysis of selected sugar porters

All potential hexose transporter genes revealing a mycorrhiza-regulated induction of gene expression compared to the extraradical mycelium (Lacbi1:304755, Lacbi1:313180, Lacbi1:301992, and Lacbi1:385212) or transcript level above one mRNA molecules per 10,000 rRNAs in ectomycorrhizas were investigated for their hexose transport properties by heterologous expression in yeast. Additionally, two highly expressed but not mycorrhizas induced genes were included into this analysis (Lacbi1:380081, Lacbi1:183424). As these six genes represent the most abundantly expressed members of the SP gene family, the potential sugar uptake capacity of *L. bicolor* hyphae in ectomycorrhizas could be estimated. Out of the six investigated genes, four (*Lacbi1:304755, Lacbi1:313180, Lacbi1:301992, Lacbi1:380081*) were capable to restore the growth defect of the yeast mutant (Fig. 8) and were renamed to LbMST1.2 (Lacbi1:313180), LbMST1.3 (Lacbi1:301992), LbMST1.4 (Lacbi1:380081), and LbMST3.1 (Lacbi1:304755).

To determine the import properties of the proteins, import studies with $^{14}C$ labeled glucose were performed. Three of the transporter proteins revealed similarly low $K_M$ values for glucose uptake (LbMST1.2: 58.6 (±2.2) µM, LbMST1.3: 64.2 (±7.5) µM), and LbMST3.1: 64.7 (±6.6) µM, respectively). The corresponding genes were all induced upon ectomycorrhiza formation. The $K_M$ value of the fourth monosaccharide transporter protein (that was not induced in ectomycorrhizas) was about seven times higher (LbMST1.4: 430.8 (±31.9) µM).

From competition experiments using radioactively labeled glucose and a 15-fold excess of a competitor sugar, a strong inhibition of fructose uptake in the presence of glucose was observed for all investigated hexose transporters. When using radioactively labeled fructose, a $K_M$ value for fructose uptake was only observed for LbMST1.2. With 1108 (±71) µM it was about 17.6 times higher than the $K_M$ value of the protein for glucose uptake. In agreement with the inability of LbMST1.4 to restore yeast growth on fructose as sole carbohydrate source, no fructose inhibition of glucose uptake was observed. Together these data clearly indicate a preference of all investigated *Laccaria* proteins for glucose uptake. As it is supposed that sucrose is exported into the common apoplast of the symbiotic interface it can be speculated that fructose would accumulate unless it is taken up by the plant partner.

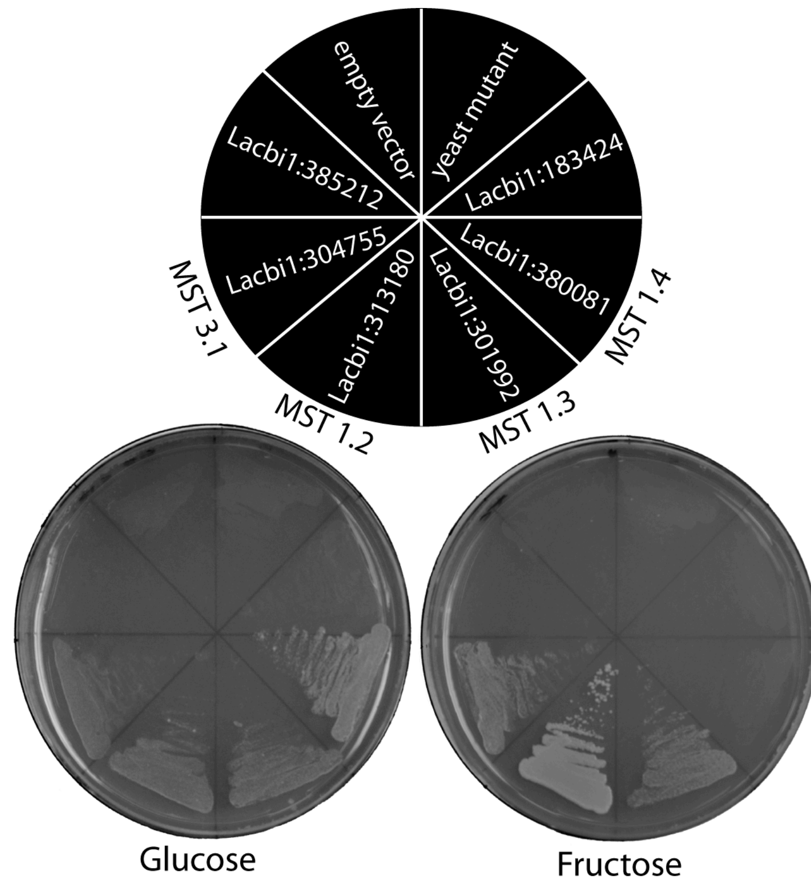



**Figure 8**: Functional complementation of a yeast mutant defective in hexose import by selected members of the *Laccaria* sugar porter gene family.

Six *Laccari*a genes coding for potential hexose transporters (Lacbi1:385212, Lacbi1:304755, Lacbi1:313180, Lacbi1:301992, Lacbi1:380081, and Lacbi1:183424) were cloned into the yeast expression vector pDR196 (Rentsch et al., 1995) and transformed together with the empty vector into the yeast mutant EBY.VW4000 (Wieczorke et al., 1999) defective in hexose import. Single yeast transformants and the non-transformed strain were analyzed for their growth on supplemented YNB medium containing either glucose or fructose as sole carbohydrate source. *Laccaria* genes that were capable of complementing the hexose import defect of the yeast mutant were renamed according to their phylogenetic affiliation.

### 5.1.6 **Summary of The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil-growing hyphae**

Based on the low sugar content in forest soils that could be a growth limiting factor in these ecosystems, the genome wide analysis of the *L. bicolor* sugar porter gene family

indicates two potential functions of sugar importers in ectomycorrhizal fungi, initially postulated on the basis of results with single hexose transporters from *A. muscaria* (Nehls, 2004; Nehls et al., 1998) and *T. borchii* (Polidori et al., 2007): 1) a reduction of carbon leakage under conditions of carbohydrate starvation and 2) the formation of a strong carbohydrate sink at the plant fungus interface in symbiosis.
Contrasting the situation in *A. muscaria* the strongly enhanced hexose uptake capacity of mycorrhizal hyphae is not regulated in a sugar-dependent manner in *L. bicolor.* Instead, developmental regulation can be supposed.

## 5.2 Increased trehalose biosynthesis in Hartig net hyphae of ectomycorrhizas

Mycorrhizal function relies on the constant supply of sugar by the host plant. Ectomycorrhizal fungi receive a significant quantity of the total carbon fixed by the plant host and thus function as an important sink for fungal hyphae. This occurs via a quick conversion of plant-derived monosaccharides into fungal metabolic compounds by Hartig net hyphae e.g. storage carbohydrates. As it is present in large quantities in well supported mycelia of certain ectomycorrhizal fungi, trehalose is supposed to act as an intermediate storage pool for imported carbohydrates. The capability to accumulate trehalose might be of special importance for hyphae of the plant/fungus interface (Hartig net). Here, large sugar amounts are imported from the common apoplast and have to be converted into fungal compounds to enable the formation of a strong fungal sink, a prerequisite for a continuous fungal carbohydrate support.

### 5.2.1 Isolation of Hartig net and sheath hyphae from established ectomycorrhizas

The compartmentalization of trehalose biosynthesis in hyphae of the Hartig net and the hyphal sheath has never been systematically studied. Hence, established ectomycorrhizas between Fly agaric (*Amanita muscaria*) and poplar (*Populus tremula x tremuloides*) were isolated and fungal sheath and Hartig net hyphae were physically separated. The fungal sheath of *A. muscaria* is only loosely attached to the apical part of infected fine roots and could be easily detached by gently lifting and tearing off using forceps. In contrast, it is tightly attached to the root surface at the middle and basal parts

of mycorrhizas, where the sheath could be separated only as small mycelial patches by inserting the tips of the forceps beneath the sheath and gently lifting. Only root-cell free patches of fungal sheath, and mycorrhizas that appeared sheath-free under the binocular microscope were used for further analysis.

### 5.2.2 cDNA and deduced protein sequences of *trehalose biosynthesis genes*

cDNA clones revealing strong homology to fungal trehalose-6-P synthase (TPS), trehalose-6-P phosphatase (TPP), and trehalose phosphorylase (TP) were identified by random sequencing of a *P. tremula* ×*tremuloides*–*A. muscaria* ectomycorrhizal cDNA library (Küster *et al*., 2007).

The *AmTPS* cDNA (accession no. AJ300447) has a length of 2311 bp and contains an open reading frame coding for a protein of 740 amino acids. The deduced protein sequence shows highest homology to TPSs from *Laccaria bicolor*, *Phanerochaete chrysosporum* and *Ustilago maydis* (60, 56, and 49% identity, respectively). Because the genome sequences of *P. chrysogenum*, *L. bicolor* and *U. maydis* contain only one *TPS* gene, it can be supposed that only a single gene copy is present in the genome of *A. muscaria*, too.

A cDNA fragment coding for a trehalose-6-P phosphatase (*AmTPP*; accession no. AJ642355) was also identified in the expressed sequence tag project (Küster *et al*., 2007). The identified clone, however, does not contain the entire reading frame of the gene.

As for other basidiomycota of the Agaricales clade a second trehalose biosynthetic pathway is present in *A. muscaria*. A single protein (trehalose phosphorylase) catalyzes both trehalose biosynthesis from glucose-1-phosphate and glucose as well as trehalose phosphorilysis. The respective *A. muscaria* gene (*AmTP)* has a length of 2310 bp and contains an open reading frame coding a protein of 696 amino acids. The deduced protein sequence of *AmTP* had highest homology to trehalose phosphorylases (TPs) from *L. bicolor* and *Pleurotus sajor-caju* (76.1 and 74.6% identity, respectively), but protein identity was always above 72% within the homobasidiomycete clade. While TP proteins of homobasidiomycetes and most filamentous ascomycetes group together in separate clusters, TPs of *Cryptococcus neoformans* (heterobasidiomycete) and *Phaeosphaeria nodorum* (ascomycete) are clearly different and as distant from other fungal TPs as the protein of an archaebacterium (*Pyrobaculum aerophilum*). As for *TPS*,

the genome of *L. bicolor* contains only one *TP* gene making it rather likely that this is true for *A. muscaria*, too (Fajardo et al., 2007).

### 5.2.3 *AmTPS*, *AmTPP* and *AmTP* expression is strongly increased in hyphae of the Hartig net

The utilization of trehalose (and/ or polyols) as a carbon store might represent a physiological adaptation of the continuous fungal sugar supply by a host plant in ectomycorrhizas. In combination with enhanced uptake of hexoses (Nehls *et al*., 1998, 2001b; Wiese *et al*., 2000) and increased metabolic turnover (Schaeffer *et al*., 1996; Kowallik *et al*., 1998; Hampp & Schaeffer, 1999; Nehls *et al*., 2001a), transformation of plant-derived monosaccharides into fungus-specific metabolites such as trehalose (Martin *et al*., 1987, 1998; Ineichen & Wiemken, 1992) could thus be the basis for the creation of a strong carbon sink in symbiosis. If this is true, trehalose biosynthesis should be favored in hyphae of the Hartig net compared to those of the fungal sheath.
Therefore, the transcript levels of genes coding for key enzymes of trehalose biosynthesis (AmTPS1, AmTPP, AmTP) were compared for hyphae of the extraradical mycelium (isolated from the same agar plate as mycorrhizas) and dissected fungal mycelia obtained from sheath and Hartig net (see Nehls *et al*., 2001a). Increased expression of all *A. muscaria* genes coding for proteins involved in trehalose biosynthesis was specifically observed for hyphae of the Hartig net. Compared to hyphae of the extraradical mycelium or of the fungal sheath the increase in transcript levels was 18-fold for *AmTPS*, 3.5-fold for *AmTPP* and four-fold for *AmTP*.

However, not only gene expression but also protein activity (trehalose-6-phosphate synthase) was increased in Hartig net hyphae upon symbiosis. Therefore, increased *AmTPS* transcript abundance (18-fold) resulted also in a significantly higher enzyme activity (7.4-fold), indicating that TPS is transcriptionally regulated in *A. muscaria.*

In contrast to *AmTPS* and *AmTPP* activity, which exclusively generate trehalose, AmTP could synthetize and degrade trehalose (Wannet, 1999). However, as Hartig net hyphae are exposed to elevated apoplastic sugar concentrations (Nehls *et al*., 2001a), also AmTP can be expected to work mainly in the direction of trehalose biosynthesis (otherwise, a futile ATP-consuming glucose/trehalose cycle would be the result).

This view is further supported by the fact that trehalose content in the Hartig net hyphae was 2.7-fold increased compared to hyphae of the fungal sheath. This (compared to gene expression and protein activity) relatively small increase in pool size may indicate a rapid trehalose export towards the extraradical mycelium, a prerequisite for both hyphal growth and perpetuation of the sink function of mycorrhizal roots. Together these data clearly show that the Hartig net is a primary site of trehalose biosynthesis in ectomycorrhizal symbiosis (Fig. 9).

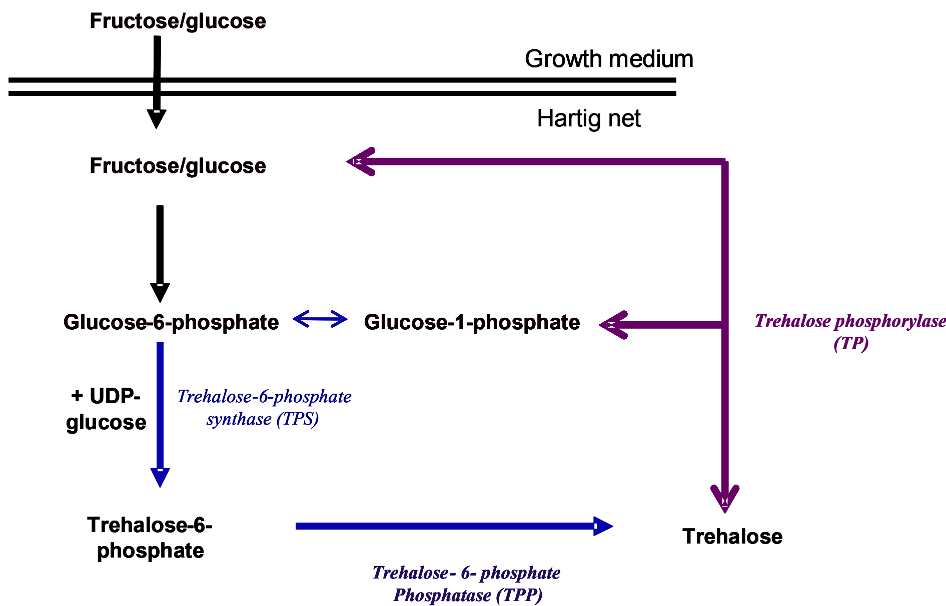



**Figure 9**: Summary of trehalose biosynthesis in the Hartig net.

### 5.2.4 Impact of sugar and nitrogen supply on *AmTPS*,*AmTPP* and *AmTP* expression

In *A. muscaria,* genes involved in sugar uptake and metabolism are regulated by external carbohydrate supply (Nehls *et al*., 2001a; Nehls, 2004). Given this impact of external hexose concentration on gene expression in *A. muscaria*, transcript levels of *AmTPS*, *AmTPP* and *AmTP* were investigated in hyphae grown in axenic liquid culture under different carbohydrate regimes. However, addition of glucose to carbon-starved mycelium showed that *AmTPS*, *AmTPP* and *AmTP* are largely unaffected by sugar supply.

Carbon metabolism and nitrogen assimilation are tightly interconnected. Therefore, nitrogen nutrition of mycelia grown in liquid culture was modulated in addition to carbon support. Nitrogen depletion resulted in a *c*. 2-fold decrease in *AmTPS* expression and a 2- to 3-fold increase in *AmTPP* transcript abundance compared with optimal growth conditions. These findings were independent of the presence or absence of a carbon source. Thus, in contrast to ectomycorrhizal symbiosis where the expression of *AmTPS* and *AmTPP* increased simultaneously in the hyphae of the Hartig net, fungal growth in pure culture on rich carbon and nitrogen sources had opposite effects on the transcript abundances of these two genes. While *AmTPS* expression was slightly induced, *AmTPP* was repressed. This can probably be explained by the finding that the substrate of TPP, trehalose-6-phosphate, acts as an important metabolic regulator in a number of fungi (Blazquez *et al*., 1993; Panneman *et al*., 1998; Foster *et al*., 2003). As increased *AmTPS* and reduced *AmTPP* activity would result in an increased cellular trehalose-6-phosphate content, fine-tuning of cellular carbon metabolism might occur via changes in the pool size of this compound in *A. muscaria*.

### 5.2.5 Expression analysis of genes involved in trehalose metabolism in *A. muscaria* fruiting bodies.

Amongst ectomycorrhizas, fruiting bodies are the second “organ” containing physiologically distinct hyphal networks, which are generated as result of a developmental program. Fruiting bodies are ephemeral structures whose major function

is to produce and disperse large numbers of generative spores. The formation of these structures is highly carbon-demanding. Carbohydrates, however, are not only the driving force for extensive cell wall formation but also for the extremely fast expansion of fruiting body primordia, which is mainly turgor driven. Fungal sugars (frequently trehalose) accumulate during this expansion process in fruiting body hyphae and drive the secondary water import into fungal cells by a strong increase in cellular osmotic pressure. As trehalose is highly mobile, the increase in trehalose content in fungal hyphae during fruiting body formation can be either explained by trehalose import from substrate mycelia or *de novo* synthesis in fruiting body cells. Therefore, gene expression of trehalose biosynthesis genes (TPS, TPP, TP) was investigated in *A. muscaria*.

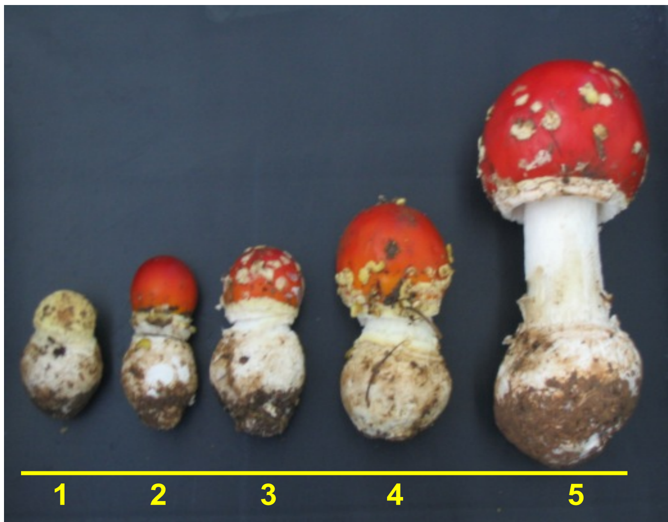
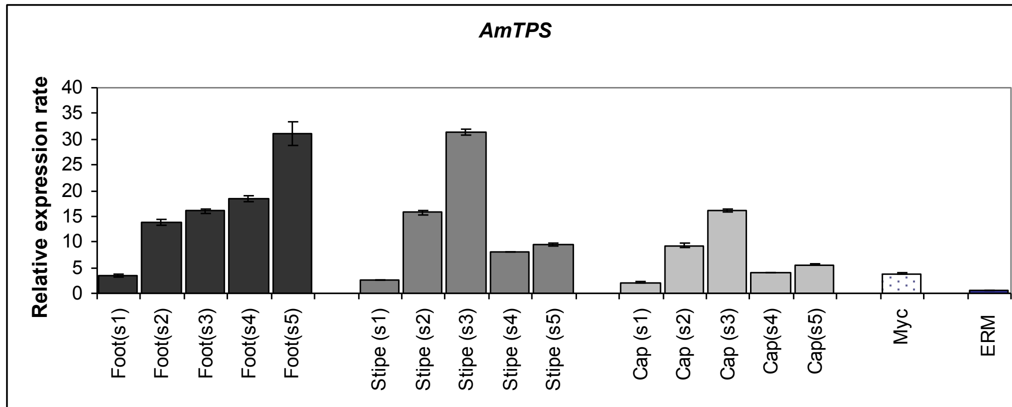
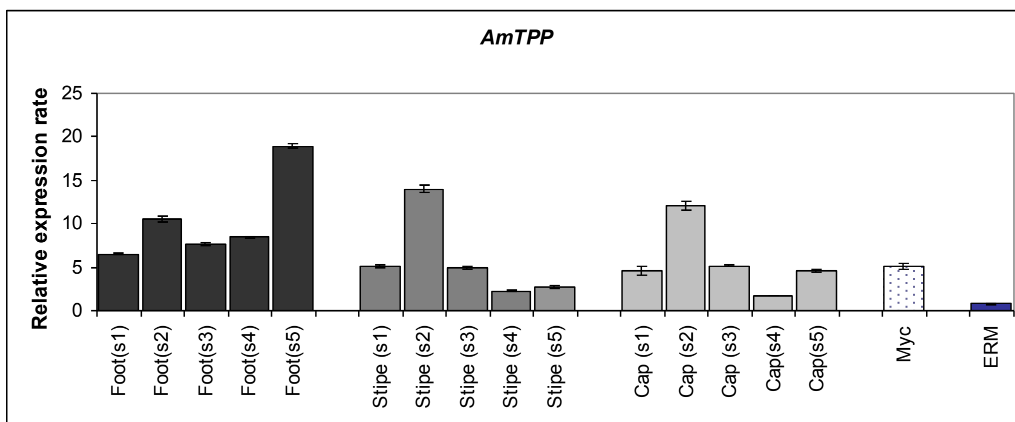



**Figure 10**: Fruiting bodies of *Amanita muscaria* analyzed in the present work.
The different expansion stages of developing fruiting bodies were assigned with numbers from 1 to 5**.**

*A. muscaria* fruting bodies corresponding to different stages of development (Fig.10) were collected from soil samples taken from spruce sites at the Schönbuch (Tübingen, Germany).

Fruiting bodies were separated in three parts: foot, stipe and cap, frozen in liquid nitrogen and stored at –80°C. Gene expression analysis was performed from first strand cDNA obtained from total RNA by quantitative RT-PCR.



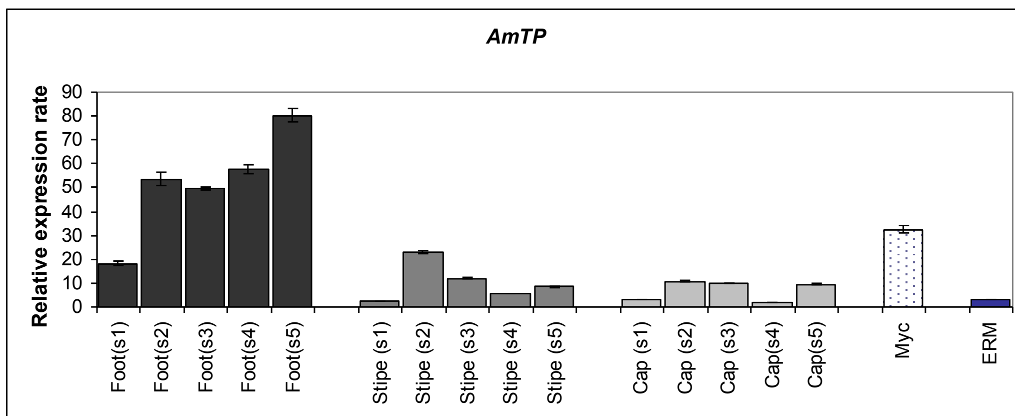




**Figure 11**: Differential expression of AmTPS, AmTPP and AmTP genes in fruiting bodies.

Total RNA was isolated from the extraradical mycelium (ERM) grown on the same agar plate as ectomycorrhizas (Myc) and foot, stipe and cap preparations of different fruiting body expansion states. Expression analysis was performed by quantitative RT-PCR using gene specific primers for AmTPS, AmTPP and AmTP. Gene expression was calibrated to the constitutively expressed fungal reference gene SCIV038 (Nehls et al. 2001a) according to Selle et al. (2005).

*AmTPS* expression was similar in cap and stipe of developing fruiting bodies (Fig.11). Transcript levels strongly increased until stage three and declined afterwards. In contrast, *AmTPS* expression constantly increased in the foot region of expanding fruiting bodies. In comparison to ectomycorrhizas *AmTPS* expression was similar at fruiting body expansion stages one, four and five (except the foot where similar transcript levels were only found at stage one). *AmTPP* transcript levels peaked in stipe and cap hyphae of stage two fruiting bodies, thus earlier than *AMTPS*. Similar to *AmTPS* expression, *AmTPP* transcript levels were highest at stage five in the foot. At most fruiting body development stages *AmTPP* expression was similar to that of ectomycorrhizas. In contrast, elevated *TP* transcript levels were mainly observed in the foot-region, where gene expression was generally much higher than in other parts of the fruiting bodies. Here, a strong increase in gene expression was found from stage two onwards. Compared to ectomycorrhizas *TP* transcript levels were lower in stipe and cap and about two-fold increased in the foot at stages two to five. (Fig.11).

Compared to the extraradical mycelium (substrate hyphae) the transcript levels of all genes encoding proteins involved in trehalose biosynthesis are strongly up-regulated at all fruiting body developmental stages. Together these expression data thus indicate an elevated trehalose biosynthetic capacity in fungal hyphae during fruiting body expansion. While stipe and cap revealed similar patterns, showing *AmTPP* expression peaks at stage two and retarded *AmTPS* peaks at stage three, highest transcripts for both genes were observed at stage 5 in the foot. TP-expression was gererally highest in the foot and increased from stage two onwards.

The formation of the complex structures of a fruit body requires major changes in the physiology of fungal hyphae (Poeggeler *et al*., 2006) and requires enormous amounts of resources that have to be delivered by the vegetative mycelium. Carbohydrates are of special importance as they are needed for biosynthetic purpose, energy formation and as osmotically active substances for turgor-driven cell elongation. Developing fruiting bodies are thus an enormous carbohydrate sink in basidiomycotic fungal colonies. The fast conversion of carbohydrates delivered by the substrate mycelium is thus a prerequisite for sink formation. As in the case of the symbiotic interface of ectomycorrhizas, trehalose formation by expanding fruit body tissues might thus be the explanation for an efficient sink formation. Gene expression data of this study are supporting previous investigations by Kitamoto and Gruen (1976). The basidiomycete

*Flammulina velutipes* showed highest incorporation of [$^{14}$C] glucose into trehalose in the fruiting body and its rapid translocation to the stipe.

### 5.2.6 **Summary of Increased trehalose biosynthesis in Hartig net hyphae of ectomycorrhizas**

In summary, in ectomycorrhizas formed between *P. tremula* × *tremuloides* and *A. muscaria* the expression of genes coding for proteins involved in trehalose biosynthesis (*AmTPS*, *AmTPP* and *AmTP*) was strongly increased upon symbiosis. Elevated transcript levels were mainly found in hyphae at the plant/fungus interface (Hartig net) but not in those of the fungal sheath. Increased fungal trehalose formation capacity occurs thus specifically at the interface where carbohydrates are released by the plant partner. Not only gene expression but also enzyme activity (AmTPS) and trehalose content are increased in hyphae of the Hartig net, indicating an elevated trehalose biosynthesis by these hyphae. Efficient carbohydrate uptake and trehalose formation together could explain the observed strong carbohydrate sink activity of mycorrhized fine roots. Carbohydrate sink formation can also be seen as major reason for the strong increase in *AmTPS*, *AmTPP* and *AmTP* gene expression in expanding fruiting bodies. A quick conversion of glucose into trehalose would reduce cellular glucose content and thus generate a steep hexose gradient between substrate mycelia and fruiting bodies that drives long distance carbohydrate transport within the fungal colony. The different kinetics of *AmTPS* and *AmTPP* expression in the foot versus stipe and hat would favor elevated trehalose formation at different expansion/developmental stages in the different parts of a fruiting body, perhaps a prerequisite for correct organ development.

# 6 Author´s contribution

**Increased Trehalose biosynthesis in Hartig net hyphae of ectomycorrhizas.** New Phytologist (2007), 174: 389-398. **Mónica Fajardo López**, Philipp Männer, Anita Willmann, Rüdiger Hampp and Uwe Nehls.

I performed 60% of this work. I set up different axcenic mycelial cultures to simulate carbohydrate and nitrogen nutrition. I was involved in mycorrhiza synthesis and sectioning. I extracted RNA from samples and performed gene expression analysis by quantitative RT-PCR.

**The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil-growing hyphae.** New Phytologist (2008), 1-14. **Mónica Fajardo López**, Sandra Dietz, Nina Grunze, Jutta Bloschies, Michael Weiß and Uwe Nehls

I performed about 50% of this work. I set the mycelia cultures with variable sources of carbon and nitrogen, took mycelial and ectomycorrhizal samples, extracted RNA and performed gene expression analysis by quantitative RT-PCR. Furthermore, I confirmed the correct amplification of full length transcripts of *L. bicolor* sugar porter genes.

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# 8 Curriculum Vitae

| | |
|---|---|
| Family Name | Fajardo López |
| Name | Mónica |
| Birth place | Bogotá, Colombia |
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Education and Research experience

| | |
|---|---|
| 06/1988-08/1995 | Bsc. In Biology. Universidad Nacional de Colombia, Bogotá, Colombia. |
| 10/1995-01/1997 | Research assistant. Biological pest control. AgrEvo. Bogotá, Colombia. |
| 02/1997-08/2001 | Research assistant. Biotechnology. Centro Internacional de Física CIF. Bogotá, Colombia. |
| 02/1998-08/2001 | Msc. Microbiology. Universidad Nacional de Colombia, Bogotá, Colombia. |
| 09/2004-08/2008 | PhD research, under the direction of PD Dr. Uwe Nehls at the Department of plant physiology and ecology, Institute of Botany, Eberhard-Karls-University. Tuebingen, Germany. |

# 9 Appendix

Primers used for analysis:

| Primer – ***Amanita muscaria*** | Sequence 5' - 3' |
|---|---|
| *SCIV038* | CTCATCTGCTCTCGTGC and GAGATAGCACCTCCACG |
| *AmTPS* | GCTGTCACAATGGATGC and AGTGCACGTTATGTAAGG |
| *AmTPP* | TGCATACCCAGGATTTG and GACACTCTACATATTCGTGC |
| *AmTP* | AATGCTGTTGCTTGGCTG and CGGTCTATAAAGTACAACAC |

| Primer – ***Laccaria bicolor*** | Sequence 5' - 3' |
|---|---|
| 18S rRNA | CAGAGCCAGCGAGTTTTTC and GTTTCCGGCTCCCCAAAGC |
| *Lacbi1:313180* | GAACTTTGGAATCGCTTATG and TGCAGCAGAAGCATGTAG |
| *Lacbi1:305352* | GTCACTTTCCACTGCGAG and AGAAGACTTTGGCCTCAAG |
| *Lacbi1:301997* | CGTCGTCAACACTGCTATG and GATGAACTCGCAGAAACAC |
| *Lacbi1:301992* | TGGGTCGTATCTCGATTG and GATGAAGTTGATGCCAGTG |
| *Lacbi1:314210* | AGTGCATCCCAATGGCTC and GCCATCGCTCCGATATTG |
| *Lacbi1:298959* | AAAGTTACGCCACAAATG and TTGGTTTCTGGTATAAGGAG |
| *Lacbi1:314197* | TTAACATCGTGGCAATGG and TGAGCTTGACGTTCCTCTG |
| *Lacbi1:306961* | GACTCCGTACCTCCAAGAG and |

| | |
|---|---|
| | AAACAGCGTCCATCTCTTC |
| *Lacbi1:300971* | ATTCCTTGGCTGTACCCTC and TGATCTCTCCCACAACCC |
| *Lacbi1:397934* | GGGCTATTACTTTCTCCTTC and AAACACCTCTCCAAGCTC |
| *Lacbi1:307251* | GGCTCTTCTACCCTGAAAC and ACCTGTATCGCCTCCTTG |
| *Lacbi1:304755* | TTTGGAACATTCAACTTTGC and AATGTGTGTCCCTGCTTG |
| *Lacbi1:142821* | GTGGTATCAACGCTCTGC and AGTTGCACGATGCCTATG |
| *Lacbi1:297020* | AACACAGCCCATACCTCC and GAGGGTATATGCGACAAC |
| *Lacbi1:317853* | GAGCTTGATTACGTCTTCG and TGTGTCCTCCTCCGAAAC |

Teile der Vorliegende Arbeit wurden bereits veröffentlicht.

**M. Fajardo, P. Männer, A. Willmann, R. Hampp and U. Nehls.** (2007). Increased Trehalose biosynthesis in Hartig net hyphae of ectomycorrhizas. New Phytologist 174: 389-398.

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# Increased trehalose biosynthesis in Hartig net hyphae of ectomycorrhizas

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## Summary

- To obtain photoassimilates in ectomycorrhizal symbiosis, the fungus has to create a strong sink, for example, by conversion of plant-derived hexoses into fungus-specific compounds. Trehalose is present in large quantities in *Amanita muscaria* and may thus constitute an important carbon sink.
- In *Amanita muscaria*–poplar (*Populus tremula* × *tremuloides*) ectomycorrhizas, the transcript abundances of genes encoding key enzymes of fungal trehalose biosynthesis, namely trehalose-6-phosphate synthase (*TPS*), trehalose-6-phosphate phosphatase (*TPP*) and trehalose phosphorylase (*TP*), were increased.
- When mycorrhizas were separated into mantle and Hartig net, *TPS*, *TPP* and *TP* expression was specifically enhanced in Hartig net hyphae. Compared with the extraradical mycelium, *TPS* and *TPP* expression was only slightly increased in the fungal sheath, while the increase in the expression of *TP* was more pronounced. TPS enzyme activity was also elevated in Hartig net hyphae, displaying a direct correlation between transcript abundance and turnover rate. In accordance with enhanced gene expression and TPS activity, trehalose content was 2.7 times higher in the Hartig net.
- The enhanced trehalose biosynthesis at the plant–fungus interface indicates that trehalose is a relevant carbohydrate sink in symbiosis. As sugar and nitrogen supply affected gene expression only slightly, the strongly increased expression of the investigated genes in mycorrhizas is presumably developmentally regulated.

**Key words:** ectomycorrhiza, enzyme activity, gene expression, trehalose, trehalose-6-phosphate-phosphatase, trehalose-6-phosphate synthase, trehalose phosphorylase.

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## Introduction

Ectomycorrhizal symbiosis is a mutualistic interaction between certain soil fungi and trees of boreal and temperate forests. It helps to overcome nutritional and carbohydrate limitations faced by the respective partners of symbiosis. The basis of this interaction is the supply of photoassimilates by the host plant.

When fungal hyphae recognize an emerging fine root of a compatible plant partner, they direct their growth towards it (Martin *et al.*, 2001) and colonize the root surface, (often) forming a sheath or mantle of hyphae, which encloses the root and isolates it from the surrounding soil (Blasius *et al.*, 1986). After or at the same time as sheath formation, fungal hyphae grow inside the infected fine root, forming highly branched structures in the apoplast of the rhizodermis (angiosperms) and in the root cortex (gymnosperms). This so-called 'Hartig net' generates a large surface area between the two partners (Kottke & Oberwinkler, 1987).

The hyphal networks of ectomycorrhizas (fungal sheath and Hartig net) have different functions (Harley & McCready, 1952; Harley & Smith, 1983; Kottke & Oberwinkler, 1987; Smith & Read, 1997). The Hartig net, which serves as an interface between plant and fungus, is adapted to the exchange of plant-derived carbohydrates for fungus-derived nutrients.

The function of the fungal sheath, in contrast, is that of intermediate storage of the nutrients that are delivered by soil-growing hyphae and are intended for delivery to the Hartig net, and of the carbohydrates that are taken up by the hyphae of the Hartig net and are intended for transport towards the soil-growing mycelium.

In ectomycorrhizal symbiosis, up to 30% of total plant photoassimilates can be transferred to the fungus to enable its proliferation (Finlay & Söderström, 1992; Söderström, 1992). Sucrose, the major long-distance transport carbohydrate of most plants, is presumably exported into the apoplast and hydrolyzed by a plant-derived acid invertase (Lewis & Harley, 1965b; Salzer & Hager, 1991; Hampp & Schaeffer, 1999). The resulting monosaccharides are then taken up by the fungal partner (Lewis & Harley, 1965a; Palmer & Hacskaylo, 1970; Chen & Hampp, 1993).

The driving force for carbon allocation in vascular plants is consumption at the sink site. Mycorrhizas attract carbohydrates much more efficiently than nonmycorrhizal fine roots (Bevege *et al.*, 1975; Cairney *et al.*, 1989), indicating a strong sink created by fungal hyphae in symbiosis. As a consequence, fungal hexose uptake capacity is greatly increased in Hartig net hyphae (Nehls *et al.*, 1998, 2001a; Wiese *et al.*, 2000; Nehls, 2004). The findings of nuclear magnetic resonance (NMR) (Martin *et al.*, 1988, 1994) and biochemical investigations (Hampp *et al.*, 1995; Schaeffer *et al.*, 1996; Kowallik *et al.*, 1998) indicate that imported hexoses are utilized for ATP generation, amino acid biosynthesis (carbon skeletons), and the formation of carbohydrate storage compounds. When not limited by carbon supply, ectomycorrhizal fungi produce a series of fungus-specific sugars and sugar alcohols (Martin *et al.*, 1985, 1987, 1988, 1998). Different pools of storage carbohydrates can be distinguished: oligosaccharides (trehalose), polyols (mannitol, arabitol and erythritol), and the long-chain carbohydrate glycogen.

In addition to its function as a carbon reserve, trehalose can also act as a stabilizer and protectant of proteins and membranes (Gadd *et al.*, 1987) against heat (Bell *et al.*, 1992), cold (Tibbett *et al.*, 2002), and oxidative stress (Banaroudj *et al.*, 2001).

In eukaryotes, trehalose can be generated by two different enzymatic routes. The combined activities of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) catalyze irreversible trehalose formation from uridine diphosphate (UDP)-glucose and glucose-6-phosphate (generating trehalose-6-phosphate as an intermediate). Trehalose phosphorylase (TP) (also known as trehalose synthase) catalyzes the reversible phosphorolysis of trehalose into glucose-1-phosphate and glucose. At cellular pH values (between 6 and 7), however, the thermodynamic equilibrium constant favors the biosynthesis of trehalose and not its degradation (Eis *et al.*, 1998).

One model system for the investigation of carbon partitioning in ectomycorrhizas is the symbiosis established between fly agaric (*Amanita muscaria*) and poplar (*Populus tremula* × *tremuloides*). Like other ectomycorrhizal fungi (Martin *et al.*, 1987, 1998; Ineichen & Wiemken, 1992), fly agaric uses trehalose as an intermediate storage pool for carbohydrates in symbiosis (Schaeffer *et al.*, 1995; Wallenda, 1996; Hampp & Schaeffer, 1999), but compartmentation of trehalose biosynthesis between hyphae of the Hartig net and the hyphal sheet has not been investigated. We thus separated physically hyphal mantle and Hartig net hyphae and studied the expression of genes coding for the key enzymes of trehalose biosynthesis: TPS, TPP and TP. In addition, we assayed the enzymatic activity of TPS, and determined the respective pool sizes of trehalose. Our data show that trehalose biosynthesis is primarily associated with Hartig net hyphae, and that it is not under the control of carbon and nitrogen supply.

## Materials and Methods

### Biological material

*Amanita muscaria* (L. Fr.) Pers. strain CS83 was isolated from a fruiting body from Schönbuch, Germany (Schaeffer *et al.*, 1995). Mycelia were grown in liquid culture or on Petri dishes for 2–16 d in modified Melin Norkrans (MMN; Marx, 1969) medium in the presence of glucose (up to 40 mM) as the carbon source and various nitrogen sources (amino acids were supplied as casein hydrolysate; N-Z Amine HD; Sigma, St Louis, MI, USA). Fungal mycelium grown in liquid culture was collected by filtration using a Büchner funnel under suction, washed twice with deionized water, frozen in liquid nitrogen, and stored at −80°C.

*Populus tremula* × *tremuloides* was used as the plant partner for mycorrhiza formation under axenic conditions according to Hampp *et al.* (1996), with MMN medium containing ammonium at a final concentration of 300 µM as the sole nitrogen source. Mycorrhizal and nonmycorrhizal fine roots and nonmycorrhizal fungal hyphae (extraradical mycelium) were harvested, frozen in liquid nitrogen, and stored at −80°C.

### Expression analysis

Expression analysis was performed by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Isolation of total RNA from samples of 80 mg fresh weight was carried out either according to Nehls *et al.* (1998) or using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Aliquots of *c.* 0.4 µg of total RNA were treated with DNAse I (Invitrogen, Carlsbad, CA, USA) and used for first-strand cDNA synthesis in a total volume of 20 µl, containing 50 pmol oligo-d(T)$_{18}$ primer (GE Healthcare Europe, Freiburg, Germany) and 200 U Superscript II RNAse H$^{-}$ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. After synthesis, 30 µl of 5 mM Tris/HCl, pH 8, was added and aliquots were stored at −80°C.

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PCR was performed in a total volume of 20 µl using 10 µl of Q-PCR-Mastermix (ABgene, Epsom, UK), 0.5 µl of cDNA, and 10 pmol of each primer in a MyiQ real-time PCR system (Bio-Rad, Hercules, CA, USA). Specific primers for the constitutively expressed *A. muscaria* genes *SCIV038* and *SCIV192* (Nehls *et al.*, 2001a) were used as reference (only the results for *SCIV038* are shown, but similar results were obtained using *SCIV192* as a reference). PCR was always performed in duplicate. At least three independent cDNA preparations were used for analysis. For the determination of PCR efficiency, dilution series of each gene were prepared and used as the PCR template. The corresponding PCR efficiencies, calculated using the MyiQ software package (version 1.0; Bio-Rad), were 91.5% for *A. muscaria* trehalose-6-phosphate synthase (*AmTPS*), 92.3% for *AmTPP*, 91% for *AmTP*, 93.8% for *SCIVO38* and 89.3% for *SCIVO192* primers, respectively.

### Primers used for analysis

*SCIV038*: 5′-CTCATCTGCTCTCGTGC-3′ and 5′-GAGATAGCACCTCCACG-3′
*SCIV192*: 5′-AAGCAGACCGTTACCTATC-3′ and 5′-TCCTGGCTCTCTTACTCC-3′
*AmTPS*: 5′-GCTGTCACAATGGATGC-3′ and 5′-AGTGCACGTTATGTAAGG-3′
*AmTPP*: 5′-TGCATACCCAGGATTTG-3′ and 5′-GACACTCTACATATTCGTGC-3′
*AmTP*: 5′-AATGCTGTTGCTTGGCTG-3′ and 5′-CGGTCTATAAAGTACAACAC-3′

### Measurement of TPS activity and trehalose content by fluorescence microscopy

Freeze-dried entire and dissected mycorrhizas (separated into fungal sheath and fine roots containing the Hartig net) as well as nondissected fine roots were cut into two or three pieces of about equal length with custom-made micro knives and weighed on glass-fiber balances (for production and calibration, see Lowry & Passonneau, 1972) in a conditioned room (40% humidity; 20°C).

The principles of measurement are described in Outlaw *et al.* (1985). The reaction cuvettes consisted of a 5-mm-thick Teflon tray with holes of 5 mm diameter. The holes were closed by a thin Teflon film (Hansa Tech, Kings Lynn, UK) at the lower end of the Teflon tray and fixed by insertion of Teflon tubing with an outer diameter identical to the diameter of the hole. The inner diameter of the tubing was 2.5 mm. To prevent evaporation of the assay solution, the wells were filled with 5 µl of purified light mineral oil (Sigma). Using glass constriction pipettes, 2 µl of assay solution was submerged in the oil. Subsequently, the tissue sample was pushed through the oil into the assay droplet by means of a tiny quartz fiber glued to a glass or wooden handle. The whole Teflon tray was then transferred to the stage of an inverted microscope (Diavert; Leitz, Bensheim, Germany). The objective lens (PL Fluotar, ×40/0.70 EF; Leitz) was focused to a layer above the Teflon membrane within the brightest area of the droplet, avoiding shadowing by the sample. The excitation light (Hg lamp, HBO 103 W/2; Leitz) passed through an excitation filter (330–380 nm) into the assay droplet. Emitted fluorescent light passed through a dichroic mirror (< 400 nm) before reaching a photomultiplier. The analog signal from the photomultiplier was digitized by an AD converter (Serial Box Interface; Vernier, Beaverton, OR, USA) and recorded using the computer program LOGGER PRO (version 2.1; Vernier).

TPS activity was assayed according to Vanderkammen *et al.* (1989; method 2). A total volume of 2 µl contained HEPES (50 mM, pH 7.6), 40 mM glucose-6-phosphate, 2 mM $MgCl_2$, 0.6 mM NADH, 1.5 mM phosphoenolpyruvate, 2 U lactate dehydrogenase, 2 U pyruvate kinase, and 1.7 mM UDP-glucose. Trehalose-6-phosphate and UDP were generated by TPS in a first reaction from UDP-glucose and glucose-6-phosphate. UDP was then phosphorylated via phosphoenolpyruvate/pyruvate kinase and the resulting pyruvate reduced to lactate under stoichiometric oxidation of NADH. The background of the reaction was measured in samples without glucose-6-phosphate.

Trehalose was quantified as glucose after hydrolysis by neutral trehalase (Jones & Outlaw, 1981). The assay consisted of 50 mM 2-morpholino-ethane-sulfonic acid (MES) (pH 6.5), 6.2 mM MgCl, 2.1 mM ATP, 0.8 mM NADP, 0.8 U glucose-6-phosphate dehydrogenase (from yeast), and 1.2 U hexokinase (from yeast). In a first step, glucose present in the tissue was converted into 6-phosphogluconic acid. When this reaction was finished, $1 \times 10^{-4}$ U of neutral trehalase (from porcine kidney; Sigma) was added, and trehalose-derived glucose determined. Fluorescence resulting from NADPH was quantified from standard curves. If not otherwise stated, chemicals and enzymes were from Roche (Mannheim, Germany).

Ergosterol, a marker for membranes of ectomycorrhiza-forming fungi, was determined by high-performance liquid chromatography (HPLC) according to Martin *et al.* (1990) and Wallenda *et al.* (1996).

### Phylogenetic analyses of AmTPS and AmTP

The predicted full-length protein sequences of AmTPS and AmTP were used for phylogenetic analysis. For alignments, full-length sequences from other fungi detected by BLASTX in the nonredundant database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/) were used. To obtain more basidiomycete sequences, publicly available fungal genomic DNA sequences from *Laccaria bicolor* (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html) and *Phanerochaete chrysogenum* (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) were screened for *TPS* and *TS* genes using BLAST X and *AmTPS* or *AmTP* as a template.

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### Miscellaneous

DNA fragments were cloned into the pCR 2.1-TOPO vector (Invitrogen) and used for transformation of One-shot competent *Escherichia coli* (Invitrogen). Overlapping sequencing was performed using M13 universal and reverse primers (Stratagene, La Jolla, CA, USA) as well as gene-specific primers (Operon Biotechnologies, Huntsville, AL, USA) and the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated ABI 3100 sequencer (Applied Biosystems) according to the manufacturer's instructions.

For analysis of DNA and protein sequences, the program packages GeneJockey II (Biosoft, Cambridge, UK) and BioEdit (version 7.0; Hall, 1999) were used. Sequence data were compared with public libraries using BlastX (http://www.ncbi.nlm.nih.gov/blast; Altschul *et al.*, 1997) and further analyzed using Clustal X (version 1.8; Thompson *et al.*, 1997).

## Results

### cDNA and deduced protein sequences of *AmTPS*, *AmTPP* and *AmTP*

cDNA clones revealing strong homology to TPSs, TPPs, and TPs were identified by random sequencing of a *P. tremula* × *tremuloides*–*A. muscaria* ectomycorrhizal cDNA library (Küster *et al.*, 2006).

The *AmTPS* cDNA (accession no. AJ300447) has a length of 2311 bp and contains an open reading frame that could code for a protein of 740 amino acids with a molecular mass of 80 167 Da. The deduced protein sequence of *AmTPS* had highest homology to TPSs from *Laccaria bicolor*, *Phanerochaete chrysosporum* and *Ustilago maydis* (60, 56, and 49% identity, respectively). TPS proteins from basidiomycetes, filamentous ascomycetes and yeast-related organisms cluster together in separate branches (Fig. 1). TPS might thus be a useful tool for phylogenetic grouping of fungi.

Southern blot analysis revealed no indication of additional *TPS* genes (data not shown), which makes it probable that *A. muscaria*, like *P. chrysogenum*, *L. bicolor* and *U. maydis*, contains only one *TPS* gene in its genome.

A cDNA fragment coding for an *AmTPP* (Accession no. AJ642355) was identified in the same expressed sequence tag (EST) project (Küster *et al.*, 2006). As the identified clone does not contain the entire reading frame, no phylogenetic analysis was carried out.

Furthermore, a cDNA coding for an *AmTP* (Accession no. AJ643200) from *A. muscaria* was identified. *AmTP* has a length of 2310 bp and contains an open reading frame that could code for a protein of 696 amino acids with a molecular mass of 77 278 Da. The deduced protein sequence of *AmTP* had highest homology to TPs from *L. bicolor* and *Pleurotus sajor-caju* (76.1 and 74.6% identity, respectively), but protein identity was always above 72% within the homobasidiomycete clade. While TP proteins of homobasidiomycetes and most filamentous ascomycetes group together in separate clusters, TPs of *Cryptococcus neoformans* (heterobasidiomycete) and *Phaeosphaeria nodorum* (ascomycete) are clearly different and as distant from other fungal TPs as the protein of an archaebacterium (*Pyrobaculum aerophilum*). As for *TPS*, the genome of *L. bicolor* also contains only one *TP* gene.

### *AmTPS*, *AmTPP* and *AmTP* expression is strongly increased in hyphae of the Hartig net

Symbiotic interaction with *Populus tremula* × *tremuloides* (Figs 2, 3) showed a 5- to 6-fold increase in *AmTPS1* transcript abundances compared with hyphae of the extraradical mycelium isolated from the same agar plate as mycorrhizas (Fig. 2). Although it was less pronounced, there was also an increase in *AmTPP* expression in mycorrhizas in comparison to free-living hyphae (*c.* 2.5-fold; Figs 2, 4). The largest differences in gene expression were obtained for *AmTP*. For this gene, transcript abundances were 25-fold higher in mycorrhizas compared with free-living (nonmycorrhizal) hyphae.

When ectomycorrhizas were separated into fungal sheath and remaining fine roots, containing the Hartig net (see Nehls *et al.*, 2001a), increased expression of all three *A. muscaria* genes was specifically observed in the hyphae of the Hartig net (Fig. 2): the increase in the amounts of transcript within Hartig net hyphae was *c.* 18-fold for *AmTPS*, 3.5-fold for *AmTPP* and 4-fold for *AmTP*. Gene expression in the hyphae of the nonmycorrhizal extraradical mycelium from the same plates was about 2-fold lower for *AmTPS* and *AmTPP* and 10-fold lower for *AmTP* compared with the hyphae of the fungal sheath (Fig. 2).

### Impact of sugar and nitrogen supply on *AmTPS*, *AmTPP* and *AmTP* expression

In light of the fact that *A. muscaria* genes involved in sugar/nitrogen uptake and metabolism have been shown to be regulated by carbohydrate and nitrogen supply (Nehls *et al.*, 2001a; Nehls, 2004), the impact of external hexoses and different nitrogen sources on expression of *AmTPS*, *AmTPP* and *AmTP* was investigated in hyphae grown in liquid culture.

The addition of glucose to carbon-starved mycelium resulted in a *c.* 5-fold increase in expression of *AmTPS* and *AmTP* after *c.* 2 h (Fig. 3). However, even after 1 d, i.e. before the glucose content in the growth medium was significantly reduced (Fig. 3, top), the transcript abundances had declined *c.* 2-fold. Sugar supply had only a minor impact on the transcript abundances of *AmTPP* (Fig. 3).

Nitrogen depletion resulted in a *c.* 2-fold decrease in *AmTPS* expression and a 2- to 3-fold increase in *AmTPP* transcript abundance compared with optimal growth conditions (Fig. 4), and these findings were independent of the presence

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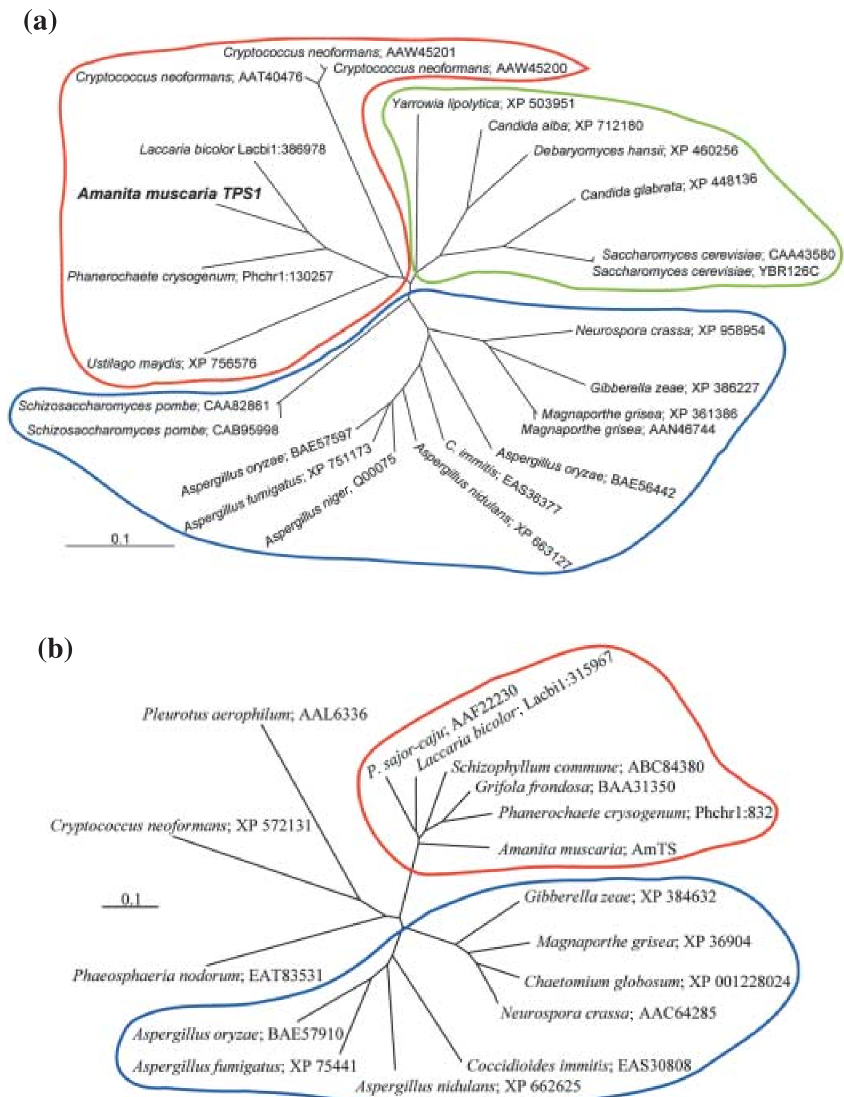


**Fig. 1** Dendrogram of the phylogenetic relationship of *Amanita muscaria* trehalose-6-phosphate synthase (AmTPS) or trehalose phosphorylase (AmTP) and related fungal proteins. The phylogenetic relationship of the deduced protein sequences of AmTPS (a) and AmTP (b) and related fungal proteins was determined by multiple sequence alignment using Clustal X (version 1.6; using default parameters) and visualized with TreeView (version 1.6.6; http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Proteins are indicated by the name of the organism and either their accession number or their unique identifier in public Joint Genome Institute (JGI) databases.

of a carbon source. In the presence of glucose, the addition of ammonium but not of amino acids resulted in a *c.* 2-fold lower transcript abundance of *AmTP*.

### *AmTPS* activity and trehalose content in hyphae of the plant–fungus interface

To address the question of whether enhanced *AmTPS* expression in hyphae of the Hartig net also results in a discernible increase in TPS activity, the hyphae of the fungal sheath and the Hartig net were analyzed separately. Because only small amounts of dissected mycorrhizal material could be obtained, a sensitive indicator reaction (NADH fluorescence) coupled to a microscope-based detection system was used (Outlaw *et al.*, 1985). Mycorrhizas were separated into the fungal sheath and the remaining fine roots (which, according to the method of Nehls *et al.* (2001a), contain the Hartig net), and freeze-dried. The Hartig net (the mycorrhiza without the hyphal mantle) contains, in contrast to the fungal mantle, only a small portion of fungal cells, so the ergosterol content was used for calibration of enzyme activity. TPS activities of 39.0 (mean value ± standard error; ± 3.1, $n = 7$) and 288.4 (± 30.4, $n = 7$) μKat $g^{-1}$ ergosterol were determined for the fungal sheath and Hartig net, respectively, indicating a 7.4-fold higher TPS activity in the hyphae of the Hartig net. No TPS activity was observed in nonmycorrhizal fine roots.

In addition to gene expression and enzyme activity, trehalose content was determined. Trehalose could be detected in entire mycorrhizas and in the fungal sheath while it was below the detection limit in the Hartig net. The trehalose content in Hartig net hyphae was thus calculated as difference between the trehalose content in entire mycorrhizas and that of the fungal sheath.

Entire mycorrhizas, the Hartig net and the fungal sheath had ergosterol contents of 0.61 (± 0.05, $n = 8$), 0.36 (± 0.07,

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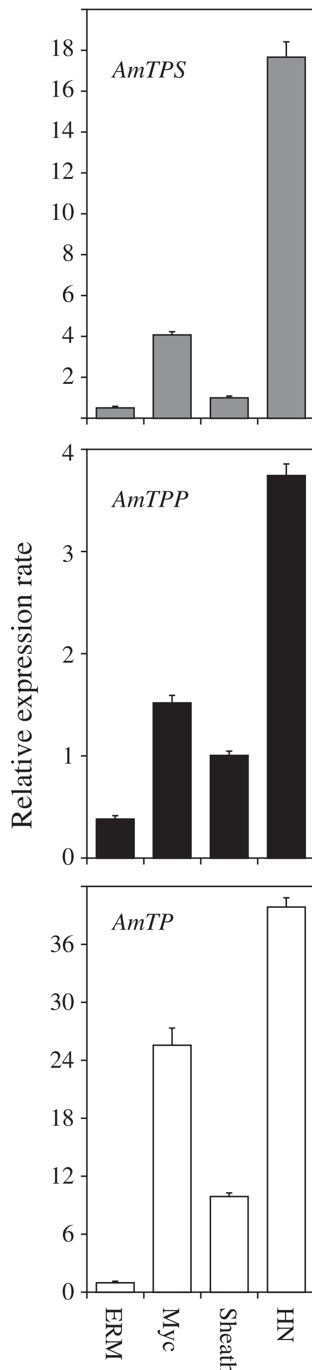


**Fig. 2** Differential expression of *Amanita muscaria* genes encoding trehalose-6-phosphate synthase (*AmTPS*), trehalose-6-phosphate phosphatase (*AmTPP*) and trehalose phosphorylase (*AmTP*) in the fungal sheath and the Hartig net of *A. muscaria* ectomycorrhizas. Total RNA was isolated from the extraradical mycelium (ERM) grown on the same agar plate as ectomycorrhizas (Myc), and ectomycorrhizas were dissected into fungal sheath (Sheath) and the remaining fine root containing Hartig net hyphae (HN). Expression analysis was performed by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using gene-specific primers for *AmTPS*, *AmTPP* and *AmTP* and calibrated to the constitutively expressed fungal reference gene *SCIV038* (Nehls *et al*., 2001a) according to Selle *et al*. (2005).

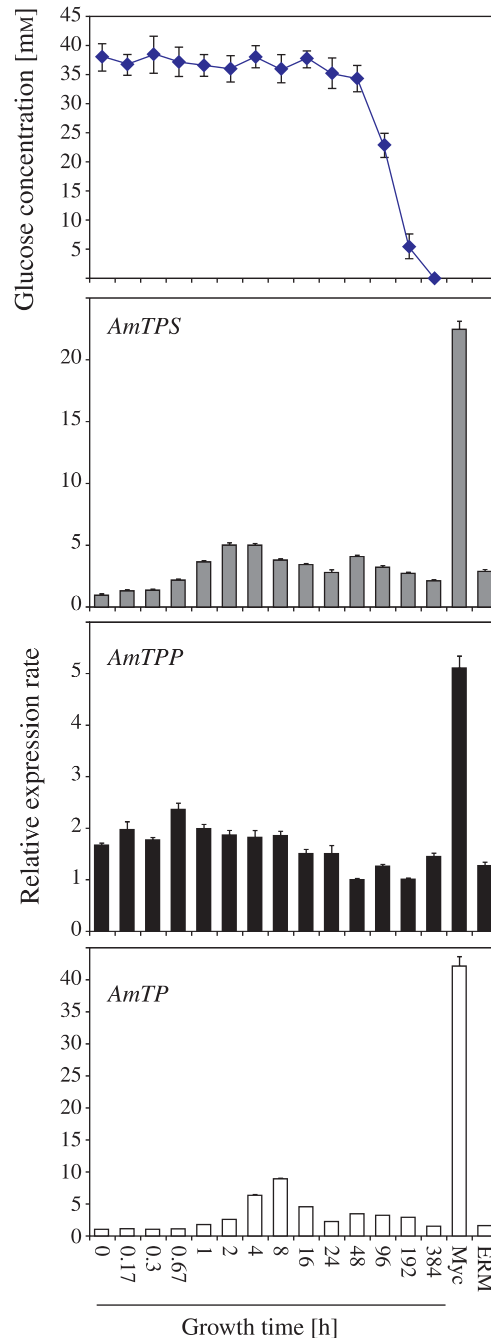


**Fig. 3** Impact of glucose nutrition on expression of *Amanita muscaria* genes encoding trehalose-6-phosphate synthase (*AmTPS*), trehalose-6-phosphate phosphatase (*AmTPP*) and trehalose phosphorylase (*AmTP*) in mycelia of *A. muscaria* grown in axenic culture. Glucose was added to a final concentration of 40 mM to mycelia precultivated in the absence of glucose for 1 wk. Mycelial samples were taken after different incubation times and actual glucose concentrations in the medium were determined. Mycorrhizas (Myc) and extraradical mycelium (ERM) were collected from the same agar plates (containing no glucose) after 6 wk of coculture. Expression analysis was performed by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using gene-specific primers for *AmTPS*, *AmTPP* and *AmTP*, and calibrated to the constitutively expressed fungal gene *SCIV038*.

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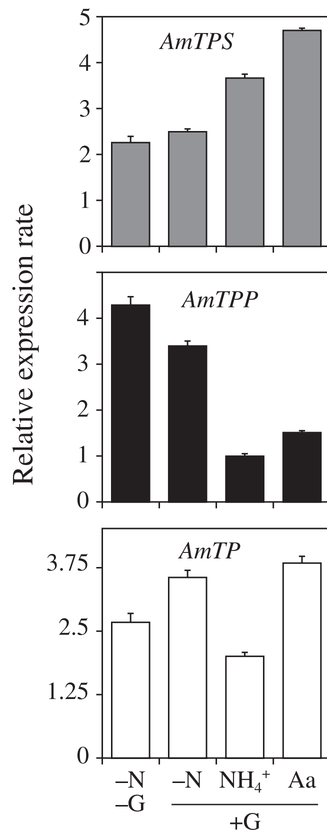


**Fig. 4** Impact of nitrogen (N) and glucose (G) nutrition on expression of *Amanita muscaria* genes encoding trehalose-6-phosphate synthase (*AmTPS*), trehalose-6-phosphate phosphatase (*AmTPP*) and trehalose phosphorylase (*AmTP*) in *A. muscaria* mycelia grown in axenic culture. Fungal mycelia were nitrogen-starved for 1 wk before addition of different nitrogen sources (samples were collected after 2 d of incubation). Expression analysis was performed by quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) using gene-specific primers for *AmTPS*, *AmTPP* and *AmTP*, and calibrated to the constitutively expressed fungal gene *SCIV038*. Aa, amino acid mixture.

$n = 7$) and 2.03 (± 0.38, $n = 7$) mg $g^{-1}$ dry weight (DW), respectively. Assuming comparable ergosterol contents in the hyphae of the fungal sheath and those of the Hartig net, entire mycorrhizas and the Hartig net (the mycorrhiza without the hyphal mantle) had fungal contents of 30 and 17.7%, respectively. This indicates a partitioning of hyphae, with 42% belonging to the fungal sheath and 58% to the Hartig net in intact *A. muscaria–P. tremula* × *tremuloides* ectomycorrhizas.

Trehalose contents were determined as 168.7 (± 27.6, $n = 9$) and 365 (± 65.6, $n = 9$) µmol $g^{-1}$ DW for entire mycorrhizas and the fungal sheath, respectively. As 17.4 and 12.6% of ectomycorrhizal DW is represented by the Hartig net and sheath hyphae, respectively, the trehalose content is *c.* 2.7 times higher in the hyphae of the Hartig net than in those of the fungal sheath (fungal sheath content per gram ectomycorrhizal DW: 365 × 0.126 = 46 µmol; Hartig net content per gram ectomycorrhizal DW: 168.7 – 46 = 122.7 µmol).

## Discussion

In saprophytic ascomycetes, trehalose was initially thought to be a storage carbohydrate in addition to glycogen. However, in contrast to glycogen, trehalose accumulates in yeast (and other ascomycetes) only during the short lag phase after the addition of glucose to a culture, and not during exponential cell growth. At least for yeast, this makes a function as a storage compound rather unlikely (Wiemken, 1990). The reason trehalose does not accumulate during exponential yeast growth is probably that high trehalase activity accompanies trehalose biosynthesis (enhanced *TPS/TPP* gene expression). As a consequence, trehalose accumulates only in trehalase-deficient yeast cells (and under stress conditions), indicating that trehalose cycling is a physiologically important feature of fast yeast growth (Francois & Parrou, 2001).

In contrast to the situation in yeast and other saprophytic ascomycetes, trehalose accumulates in many ectomycorrhizal fungi growing on glucose and in symbiosis (Martin *et al.*, 1988; Ineichen & Wiemken, 1992; Wallenda, 1996; Wallenda *et al.*, 1996; Smith & Read, 1997; Rangel-Castro *et al.*, 2002), independent of their classification as ascomycetes or basidiomycetes. In ectomycorrhizal fungi, which show a relatively slow cell division rate, the utilization of trehalose (and/or polyols) as a carbon store could therefore be a physiological adaptation to slow growth together with a continuous sugar supply from a host plant. In combination with enhanced uptake of hexoses (Nehls *et al.*, 1998, 2001b; Wiese *et al.*, 2000) and increased metabolic turnover (Schaeffer *et al.*, 1996; Kowallik *et al.*, 1998; Hampp & Schaeffer, 1999; Nehls *et al.*, 2001a), transformation of plant-derived monosaccharides into fungus-specific metabolites such as trehalose (Martin *et al.*, 1987, 1998; Ineichen & Wiemken, 1992) could thus be the basis for the creation of a strong carbon sink, which is necessary to allocate photoassimilates towards the mycorrhizal root.

'Metabolic zonation' and 'physiological heterogeneity' have been discussed as important concepts for a functional understanding of ectomycorrhizal symbiosis (Cairney & Burke, 1996; Timonen & Sen, 1998; Nehls *et al.*, 2001b). In *A. muscaria* ectomycorrhizas, *AmTPS* and *AmTPP* expression was induced almost exclusively in the hyphae of the Hartig net. Here, increased *AmTPS* transcript abundances (18-fold) resulted in significantly higher enzyme activity (7.4-fold), indicating that TPS activity is presumably transcriptionally regulated in *A. muscaria*.

Together with that of *AmTPS*, *AmTPP* expression was also up-regulated. Because of the high trehalose background we were, however, not able to properly assay TPP activity, but it can be assumed that the latter is also increased.

As in the case of *AmTPS* and *AmTPP*, *AmTP* transcript abundances were much higher in Hartig net hyphae than in those of the fungal sheath. However, in contrast to AmTPS and AmTPP activity (exclusively generating trehalose), AmTP could work in both directions, in the biosynthesis and the

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phosphorolysis of trehalose (Wannet, 1999). As Hartig net hyphae are exposed to elevated apoplastic sugar concentrations (Nehls *et al.*, 2001a), the greatly enhanced expression of all three genes indicates that AmTP is mainly active in the direction of trehalose biosynthesis (otherwise, a futile ATP-consuming glucose/trehalose cycle would be the result). In contrast to those of the Hartig net, the hyphae of the fungal sheath are not exposed to elevated apoplastic sugar concentrations and are thus dependent on the utilization of endogenous resources. As trehalose phosphorolysis would reduce the energy demand for trehalose utilization, it could be speculated that in hyphae of the fungal sheath AmTP catalyzes trehalose degradation to reduce the energy demand of these fungal cells. This speculation is supported by the observation that, in comparison to the extramycelial mycelium, expression of *AmTPS* and *AmTPP* was only slightly (2-fold) enhanced in the fungal sheath, while *AmTP* revealed 10-fold higher transcript abundances. A development-dependent regulation of fungal TP activity was demonstrated for *Agaricus bisporus*. In substrate mycelia, which were well supplied with external carbohydrates, TP catalyzed trehalose formation. However, in hyphal aggregates (developing fruit body primordia), TP degraded trehalose, yielding glucose and glucose-1-phosphate as carbon and energy sources (Wannet, 1999). Similar to the situation in *A. bisporus*, *TP* seems also to be developmentally regulated in *A. muscaria*.

In addition to gene expression and enzyme activity, trehalose content in the Hartig net was increased 2.7-fold in relation to the hyphae of the fungal sheath. This relatively small increase in pool size could be a result of rapid turnover and/or export towards the extraradical mycelium, a prerequisite for both hyphal growth and perpetuation of the sink function of mycorrhizal roots. Independent of the fate of trehalose, the data clearly show that the Hartig net is the primary site of trehalose biosynthesis in ectomycorrhizal symbiosis.

Differences in the apoplastic hexose concentrations of the Hartig net and the fungal sheath (Nehls *et al.*, 2001a,b) as well as in their cellular nitrogen contents (Nehls *et al.*, 2001a; Wipf *et al.*, 2002; Javelle *et al.*, 2004; Nehls, 2004) have been shown to act as signals regulating fungal physiological heterogeneity in ectomycorrhizas. This is obviously less relevant for the genes in this study. Compared with symbiosis, external glucose concentrations and the cellular nitrogen content had only a minor impact on the expression of any of the investigated genes and are thus obviously not the relevant signals. Evidently, signals other than sugar and nitrogen concentrations exist for the regulation and maintenance of physiological heterogeneity in fly agaric–poplar ectomycorrhizas.

In contrast to ectomycorrhizal symbiosis, where the expression of *AmTPS* and *AmTPP* increased simultaneously in the hyphae of the Hartig net, fungal growth on rich carbon and nitrogen sources in pure culture had opposite effects on the transcript abundances of the two genes. While *AmTPS* expression was slightly induced under these conditions, that of *AmTPP* was repressed. This can probably be explained by the finding that the substrate of TPP, trehalose-6-phosphate, acts as an important metabolic regulator in a number of fungi (Blazquez *et al.*, 1993; Panneman *et al.*, 1998; Foster *et al.*, 2003). As increased *AmTPS* and reduced *AmTPP* activity would result in an increased cellular trehalose-6-phosphate content, fine-tuning of cellular carbon metabolism might occur via changes in the pool size of this compound in *A. muscaria* also.

In summary, in ectomycorrhizas formed between *A. muscaria* and *P. tremula* × *tremuloides*, expression of genes coding for proteins involved in trehalose biosynthesis (*AmTPS*, *AmTPP* and *AmTP*), as well as TPS activity and trehalose content, are increased specifically in hyphae located at the plant–fungus interface. This indicates that it is the Hartig net that is responsible for both the creation of the carbon sink and the redistribution of plant-derived carbohydrates. As AmTP can catalyze both biosynthesis and degradation of trehalose, and as its activity is under metabolite control, investigation of metabolite pools in the hyphae of the fungal sheath and the Hartig net will be the aim of future work.

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# The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil-growing hyphae

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## Summary

- Formation of ectomycorrhizas, a symbiosis with fine roots of woody plants, is one way for soil fungi to overcome carbohydrate limitation in forest ecosystems.
- Fifteen potential hexose transporter proteins, of which 10 group within three clusters, are encoded in the genome of the ectomycorrhizal model fungus *Laccaria bicolor*. For 14 of them, transcripts were detectable.
- When grown in liquid culture, carbon starvation resulted in at least twofold higher transcript abundances for seven genes. Temporarily elevated transcript abundance after sugar addition was observed for three genes. Compared with the extraradical mycelium, ectomycorrhiza formation resulted in a strongly enhanced expression of six genes, of which four revealed their highest observed transcript abundances in symbiosis. A function as hexose importer was proven for three of them. Only three genes, of which just one was expressed at a considerable level, revealed a reduced transcript content in mycorrhizas.
- From gene expression patterns and import kinetics, the *L. bicolor* hexose transporters could be divided into two groups: those responsible for uptake of carbohydrates by soil-growing hyphae, for improved carbon nutrition, and to reduce nutrient uptake competition by other soil microorganisms; and those responsible for efficient hexose uptake at the plant–fungus interface.

**Key words:** basidiomycetes, ectomycorrhiza, fungi, sugar porter gene family, sugar transport.

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## Introduction

Although litter and humus layers of forest soils are quite rich in complex carbon sources (e.g. cellulose and lignin), most ectomycorrhizal (EM) fungi seem to be dependent on simple, readily utilizable carbohydrates. The reason for this is that EM fungi have, compared with wood and litter decomposers, only a limited degradation capability (Colpaert & van Tichelen, 1996; Read & Perez-Moreno, 2003).

In contrast to forest soils where simple carbohydrates are rare (Wainwright, 1993), plant root exudates can be rich in simple carbohydrates. The strategy of EM fungi to face their carbohydrate limitation is a tight association with fine roots of woody plants, forming an interindividual organ, the ectomycorrhiza. Here, EM fungi have direct and privileged access to root exudates both because the root surface is covered by a sheath of hyphae and fine roots are thus isolated from the surrounding soil; and because fungal hyphae grow inside the infected fine root, forming highly branched structures in the apoplast, the so-called Hartig net (Blasius *et al.*, 1986), to enable nutrient and metabolite exchange with the host.

Furthermore, because fungi contribute to tree nutrition (Landeweert *et al.*, 2001; Read & Perez-Moreno, 2003), EM roots gain much more carbon than do nonmycorrhizal plant roots (Bevege *et al.*, 1975; Cairney *et al.*, 1989; McDowell *et al.*, 2001). Percentages as high as 47–59% of plant photosynthates are allocated to ectomycorrhizas (McDowell *et al.*, 2001) and part of this surplus in carbohydrates is transferred towards the

fungal partners. Several authors estimate that 20–25% of the net photosynthesis rate is used for fungal support (Söderström, 1992; Högberg & Högberg, 2002; Hobbie, 2006). As a consequence of the elevated carbon demand in symbiosis, host plants increase their carbon fixation rates (Lamhamedi *et al.*, 1994; Loewe *et al.*, 2000; Wright *et al.*, 2000).

Carbon compounds delivered by the plant partner in symbiosis are most likely soluble sugars (for reviews, see Smith *et al.*, 1969; Harley & Smith, 1983; Smith & Read, 1997). The creation of a strong carbohydrate sink by the fungus, such as observed in EM symbiosis, is directly related to the efficiency of fungal hexose uptake at the plant–fungus interface (Nehls *et al.*, 2001b; Nehls, 2004; for a review see Nehls, 2008). While more than 20 functional sugar transporters are known from *Saccharomyces cerevisiae* (Boles & Hollenberg, 1997), only a very small number of transporters (two from *A. muscaria* (Nehls *et al.*, 1998; Nehls, 2004) and one from *Tuber borchii* (Polidori *et al.*, 2007)) have been investigated from EM fungi so far. Thus, there is a large gap in our knowledge about hexose import into EM fungi. To fill this gap, we have identified genes encoding putative sugar transporters from the recently sequenced genome of the EM fungus *Laccaria bicolor* and investigated the expression of the whole gene family and transport properties of selected members.

## Materials and Methods

### Biological material

*Laccaria bicolor* (Maire) P.D. Orton (strain S238N) mycelia were grown on Petri dishes or in liquid culture for up to 16 d with MMN (Modified Melin Norkrans; Marx, 1969) medium in the presence of different carbon sources (at final concentrations of up to 10 mM), sugar analogs and various nitrogen sources (final concentration of 300 µM). Mycelia grown in liquid culture were collected by filtration using a Büchner funnel under suction, washed twice with deionized water, frozen in liquid nitrogen, and stored at –80°C.

*Populus tremula* × *tremuloides* was used as plant partner for mycorrhiza formation under axenic conditions according to Hampp *et al.* (1996), with MMN medium containing no sugar and ammonium at a final concentration of 300 µM as sole nitrogen source. Mycorrhized and nonmycorrhized fine roots and nonmycorrhizal fungal hyphae (extraradical mycelium) were harvested, frozen in liquid nitrogen, and stored at –80°C.

### Expression analysis

Expression analysis was performed by quantitative RT-PCR. Isolation of total RNA was carried out either according to Nehls *et al.* (1998) or by using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA removal and first-strand cDNA synthesis was performed using 0.1 µg total RNA and the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. After synthesis, 30 µl of 5 mM Tris/HCl, pH 8, were added and aliquots were stored at –80°C.

Polymerase chain reaction was performed in a total volume of 20 µl using 10 µl Q-PCR-Master mix (ABgene, Epsom, UK), 1 µl cDNA and 10 pmol of each primer in a MyiQ real-time PCR system (BioRad, Hercules, CA, USA). Specific primers for *L. bicolor* 18S rRNA were used as references. PCR was always performed in duplicates. At least three independent cDNA preparations were used for analysis. For quantification, dilution series of photometrically quantified DNA fragments of each gene and the references were prepared and used as the PCR template together with first-strand cDNA samples. PCR efficiencies, as calculated by the MyiQ software package (Version 1.0, BioRad), were between 85 and 95%.

Primers used for expression analysis (names refer to the protein IDs found in the *L. bicolor* genome database v1.0 at http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html) are as follows: 18S rRNA, 5′-CAGAGCCAGCGAGTTTTTC-3′ and 5′-GTTTCCGGCTCCCCAAAGC-3′; *Lacbi1:313180*, 5′-GAACTTTGGAATCGCTTATG-3′ and 5′-TGCAGCAGAAGCATGTAG-3′; *Lacbi1:305352*, 5′-GTCACTTTCCACTGCGAG5′-3′ and 5′-AGAAGACTTTGGCCTCAAG-3′; *Lacbi1:380081*, 5′-CGTCGTCAACACTGCTATG-3′ and 5′-GATGAACTCGCAGAAACAC-3′; *Lacbi1:301992*, 5′-TGGGTCGTATCTCGATTG-3′ and 5′-GATGAAGTTGATGCCAGTG-3′; *Lacbi1:314210*, 5′-AGTGCATCCCAATGGCTC-3′ and 5′-GCCATCGCTCCGATATTG-3′; *Lacbi1:298959*, 5′-AAAGTTACGCCACAAATG-3′ and 5′-TTGGTTTCTGGTATAAGGAG-3′; *Lacbi1:183424*, 5′-TTAACATCGTGGCAATGG-3′ and 5′-TGAGCTTGACGTTCCTCTG-3′; *Lacbi1:306961*, 5′-GACTCCGTACCTCCAAGAG-3′ and 5′-AAACAGCGTCCATCTCTTC-3′; *Lacbi1:300971*, 5′-ATTCCTTGGCTGTACCCTC-3′ and 5′-TGATCTCTCCCACAACCC-3′; *Lacbi1:397934*, 5′-GGGCTATTACTTTCTCCTTC-3′ and 5′-AAACACCTCTCCAAGCTC-3′; *Lacbi1:191542*, 5′-GGCTCTTCTACCCTGAAAC-3′ and 5′-ACCTGTATCGCCTCCTTG-3′; *Lacbi1:304755*, 5′-TTTGGAACATTCAACTTTGC-3′ and 5′-AATGTGTGTCCCTGCTTG-3′; *Lacbi1:142821*, 5′-GTGGTATCAACGCTCTGC-3′ and 5′-AGTTGCACGATGCCTATG-3′; *Lacbi1:297020*, 5′-AACACAGCCCATACCTCC-3′ and 5′-GAGGGTATATGCGACAAC-3′; *Lacbi1:385212*, 5′-GAGCTTGATTACGTCTTCG-3′ and 5′-TGTGTCCTCCTCCGAAAC-3′.

Even when located within the coding region, primer pairs were chosen to be specific for each member of the gene family by primer sequence alignment to the *L. bicolor* genome sequence. To prove the amplification of the correct member of the gene family, purified PCR products originating from expression analysis were used for direct sequencing.

The expression level of the different members of the sugar porter (SP) gene family varied over a range of about five orders

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of magnitude. As protein content of hexose transporters is correlated to the transcript abundance, and abundant proteins contribute more to the overall transport properties of hyphae, genes were artificially grouped by their expression strength. Genes revealing a transcript abundance above 1 mRNA molecule per 10 000 rRNA molecules in any of the investigated conditions were called strongly expressed.

### Heterologous expression of selected sugar transporter genes

Entire coding regions were PCR-amplified from first-strand cDNA using gene-specific primers and the Phusion Taq polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions, and cloned into the pJET1/blunt vector using the GeneJET PCR Cloning Kit (Fermentas, Vilnius, Lithuania). Primers used for amplification of selected genes were as follows: *Lacbi1:313180*, 5′-ACCGACCATGCC-AGGAGG-3′ and 5′-AATTAGGCCTTCTCATCAC-3′; *Lacbi1:301992*, 5′-CATACGACGTAATGGGTG-3′ and 5′-ATTTGAAAGATCAGTTCTCG-3′ *Lacbi1:380081*, 5′-CTACTATAACAATGGGTGGAG-3′ and 5′-CGTTT-TCTTGCACCTACACAC-3′; *Lacbi1:183424*, 5′-TCCTCA-ACGATGGCTGTC-3′ and 5′-AATCATTAAGCCTTCT-CAGC-3′; *Lacbi1:304755*, 5′-CATTGTAGTATCGACATGG-3′ and 5′-TATGGGACCGAATCATGC-3′; *Lacbi1:385212*, 5′-CTCGTCTTTCGCAATGTCC-3′ and 5′-TTCTCC-ACAATCACCTACG-3′.

The correct PCR amplification of the coding region was proven by sequencing using vector as well as internal gene-specific primers. Only clones containing cDNAs identical to the *Laccaria* genome sequence were further used.

For functional analysis of the proteins encoded by the respective genes, cDNA fragments containing the entire coding region were cloned into the yeast expression vector pDR196 (Rentsch *et al.*, 1995) followed by sequencing of the cDNA/vector junctions to prove the correct insertion of the cDNAs. Two strategies were followed:

- Direct digestion of cloned cDNA fragments using peripheral or vector-located restriction enzyme recognition sites. *Lacbi1:313180* and *Lacbi1:301992* cDNA fragments were released using Kpn2I/PstI (Fermentas) double digestion and ligated into the XmaI- (NEB, Beverly, MA, USA) and PstI (Fermentas)-digested pDR vector using T4-DNA ligase (Fermentas) according to the manufacturer's instructions. *Lacbi1:183424* was excised by XbaI/XhoI (Fermentas) double digestion and ligated into the SpeI/XhoI (Fermentas) digested pDR-vector.
- Introduction of new, unique restriction enzyme recognition sites by a second round of PCR amplification. The open reading frames of *Lacbi1:385212*, *Lacbi1:304755* and *Lacbi1:380081* were amplified using Phusion Taq polymerase (Finnzymes) and primers introducing a 5′-SpeI-site and a 3′-SalI-site. Primers used for amplification were as follows: *Lacbi1:385212*, 5′-CTTACTAGTCTTTCGCAATGTCC-3′ and 5′-TAT-GTCGACTCTCCACAATCACCTACG-3′; *Lacbi1:304755*, 5′- CATACTAGTATCGACATGG-3′ and 5′-TATGTC-GACCGAATCATGC-3′; *Lacbi1:380081*, 5′-TATACTAG-TATAACAATGGGTGGAG-3′ and 5′-ATAGTCGACGT-TTTCTTGCACCTACACAC-3′.

The correct PCR amplification was checked by sequencing. After SpeI/SalI (Fermentas) double digestion, the DNA fragments were ligated into the similarly digested pDR196 vector.

The *S. cerevisiae* strain EBY.VW4000 (Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3; Wieczorke *et al.*, 1999) was transformed with the constructs and the empty pDR196 vector by electroporation with chemical pretreatment (Thompson *et al.*, 1998). Uracil auxotrophic transformants were identified by growth at 30°C on 2% agar plates consisting of 0.67% yeast nitrogen base with ammonium sulfate supplemented with leucine, tryptophane, histidine and 2% maltose.

### Uptake experiments

Uptake experiments were performed according to Doehlemann *et al.* (2005). Yeasts were grown in YNB 2% maltose to an $OD_{600}$ = 0.5–0.8, harvested by centrifugation, washed twice with potassium phosphate buffer (pH 5) and resuspended in the same buffer to an $OD_{600}$ of approx. 10. Uptake experiments were started by mixing 100 µl yeast suspension with 100 µl of radioactive-labeled sugars (Amersham, Braunschweig, Germany; specific activity, 10–400 kBq $\mu mol^{-1}$). Samples were taken after 1, 2, 5 and 7 min.

For competition experiments, mixtures of radioactive-labeled glucose (final concentration 0.17 and 0.8 mM) and one non-radioactive sugar (only D-sugars and -glucose analogs were used, with final concentrations of 2.6 and 16 mM, respectively; Sigma, Deisenhofen, Germany) in a total volume of 100 µl were added to 100 µl yeast suspension. Samples were taken after 1, 2, 5 and 7 min. At least three different experiments with three replicates each were performed. $K_m$ values were calculated using the Hyper-software (John Easterby's Software, http://www.liv.ac.uk/~jse).

### Construction of the phylogenetic tree

Gene models of the *L. bicolor* (strainS238N-H82) genome (Martin *et al.*, 2008), as predicted by the JGI (available at http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html) using four different methods (GENEWISE, FGENESH, GRAILEXP6, and EUGENE), were employed as a basis for the identification of putative hexose transporter genes. All gene models were manually inspected and the best-fitting protein (based on sequence alignment with known proteins from other organisms and cDNA sequencing) was used for analysis. Additionally, the genome sequence was screened for further potential monosaccharide transporter (MST) genes using BlastN and tBlastN and, as a template, fungal sugar

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importers with proven hexose transport capabilities (all *S. cerevisiae* transporters, Wieczorke *et al.*, 1999; *Am*MST1, *Amanita muscaria*, Nehls *et al.*, 1998; *BcFRT1*, *Botrytis cinerea*, Doehlemann *et al.*, 2005; *TBHXT1*, *Tuber borchii*, Polidori *et al.*, 2007).

Deduced amino acid sequences were aligned with DIALIGN (Morgenstern, 1999). For phylogenetic reconstruction, those positions that received scores as low as 0, 1 or 2 were excluded from the alignment. With the reduced alignment we ran maximum-likelihood analyses (Felsenstein, 1981) with the RAxML software (Stamatakis, 2006) over 100 rounds of heuristic search, using the JTT model of amino acid substitution (Jones *et al.*, 1992) and accounting for heterogeneity in substitution rates using the PROTMIX approach (Stamatakis, 2006), according to which 25 discrete substitution rates were implemented during heuristic search and the final tree was optimized using the JTT+Gamma model. To derive branch support values, 200 rounds of nonparametric bootstrap analysis (Felsenstein, 1981) were run with RAxML with the same substitution model as indicated above, with one heuristic search in each bootstrap replicate. The tree was rooted using the *Arabidopsis* proteins as an out group.

### Miscellaneous

Sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an automated ABI 3130 sequencer (Applied Biosystems) according to the manufacturer's instructions.

For analysis of DNA and protein sequences, the program package Gene Jockey II (1998, P.I. Taylor, Cambridge, UK) was used. The DNA sequence information was compared with publicly available sequence information using BlastX (NCBI, http://www.ncbi.nlm.nih.gov/blast; Altschul *et al.*, 1997). For statistical analysis Student's *t*-test was used.

## Results

### Phylogenetic relationships of *L. bicolor* sugar transporters

A total of 15 potential candidate genes were identified by searching the JGI website (available at http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html) for annotated putative hexose transporter genes and further screening of the genome sequence by using BlastN and tBlastN and fungal sugar importers with proven hexose import capability (Nehls *et al.*, 1998; Wieczorke *et al.*, 1999; Doehlemann *et al.*, 2005; Polidori *et al.*, 2007) as a template. Similar gene numbers as in *L. bicolo*r were found in the genomes of *Coprinopsis cinerea* (18; http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html), *Phanerochaete chrysosporium* (16; Martinez *et al.*, 2004), *Ustilago maydis* (19; Kämper *et al.*, 2006), *Aspergillus niger* (20; Pel *et al.*, 2007) and *Saccharomyces cerevisiae* (20; Boles & Hollenberg, 1997). A significantly larger number was found only in *Cryptococcus neoformans* (Loftus *et al.*, 2005), with 48 potential sugar transporter genes.

For phylogenetic analysis (based on deduced protein sequences), gene models of all identified *L. bicolor* hexose transporters were manually inspected and the best-fitting protein (based on sequence alignment with known proteins from other organisms and cDNA sequencing) was used. From six genes (*Lacbi1:304755*, *Lacbi1:313180*, *Lacbi1:301992*, *Lacbi1:385212*, *Lacbi1:380081*, *Lacbi1:183424*), the corresponding cDNAs were amplified for functional analysis (see below). For one of these genes (*Lacbi1:301992*), the cDNA sequence differed from the best predicted model and the corrected protein sequence (accession no. AM998533) was used for phylogenetic analysis.

According to our analysis, the fungal SP proteins (Saier, 2000) are more closely related to a group of human sugar transporters than to those of plants (Supplementary material, Fig. S1; *Arabidopsis* proteins were chosen for the alignment). However, only one fungal (*Ustilago maydis*) protein clustered together with these human SP proteins.

Ten out of the 15 *L. bicolor* identified putative SP proteins fell into three different clusters supported by bootstrap values above 60% (Fig. 1). Two members each (cluster 1: Lacbi1:301992, Lacbi1:380081; cluster 3: Lacbi1:183424, Lacbi1:314210) turned out to be not only highly homologous regarding their protein sequences but also physically linked on a single scaffold. It can thus be supposed that these genes are the result of recent gene duplications.

The four deduced *L. bicolor* proteins of cluster 1 cluster together with 19 out of 20 *Saccharomyces* members of the SP gene family and both EM fungal sugar transporters that have been functionally characterized to date (TBHXT1 from *Tuber borchii* (Polidori *et al.*, 2007) and AmMST1 from *Amanita muscaria* (Nehls *et al.*, 1998)). These data thus indicate that these *L. bicolor* proteins could be supposed as functional hexose transporters. With the exception of members of this protein cluster, only two further fungal proteins of the SP gene family have been successfully functionally characterized to date. STL1 from *S. cerevisia*e (which clusters together with Lacbi1:191542) was shown to be a glycerol transporter (Ferreira *et al.*, 2005), while BcFRT1from *Botrytis cinerea* (which clusters together with Lacbi1:385212) was characterized as a fructose importer (Doehlemann *et al.*, 2005).

### Impact of carbon nutrition on sugar transporter gene expression

In the EM fungus, *A. muscaria* gene expression is influenced by external sugar supply (Nehls *et al.*, 1998, 2001a, 2007). To investigate the impact of carbohydrate nutrition on the expression of potential hexose transporter genes in *L. bicolor*, mycelia were pre-cultivated in liquid culture in the absence of

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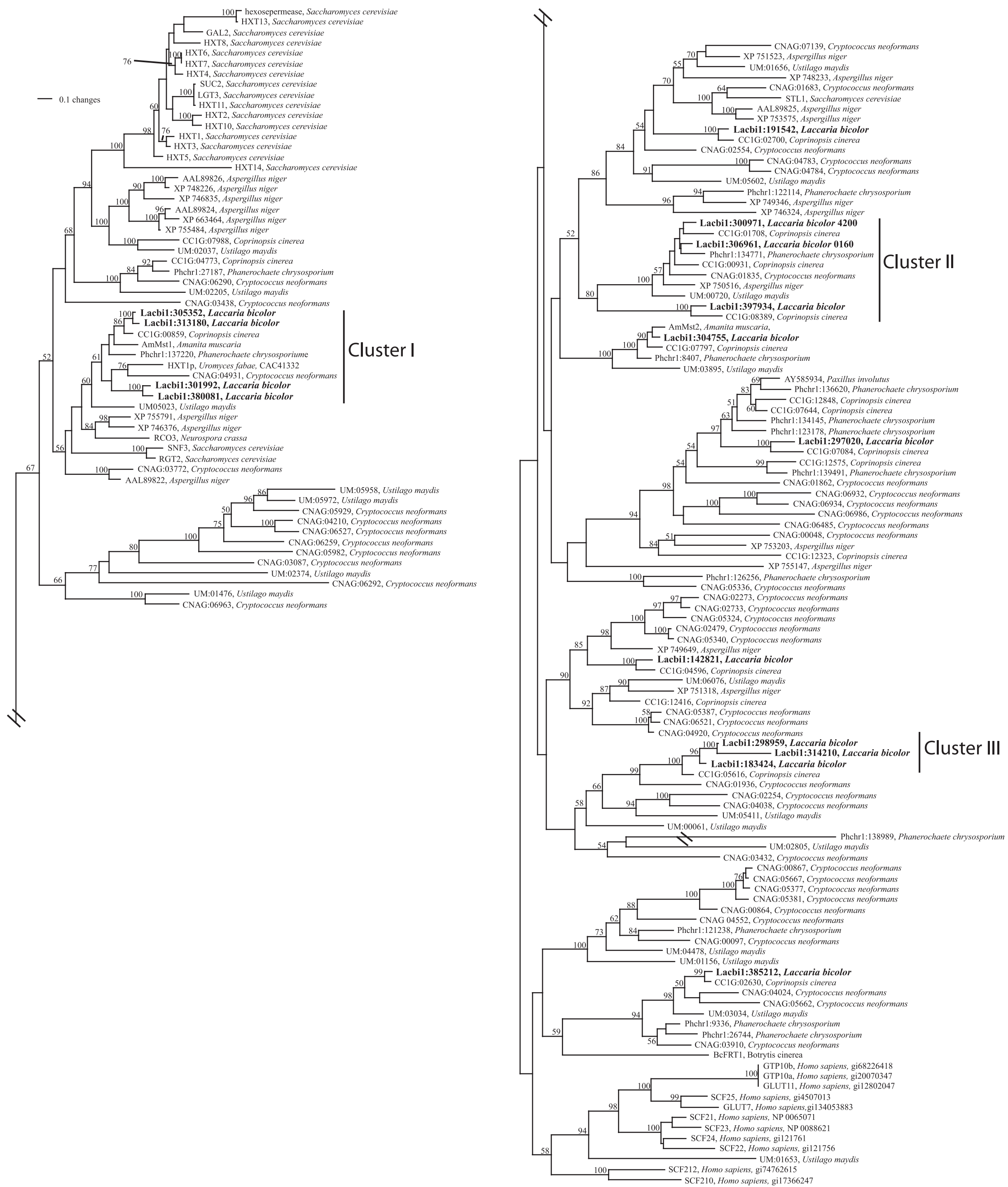


**Fig. 1** Phylogenetic relationships of the deduced protein sequences of the *Laccaria* sugar porter gene family with known fungal hexose transporters. Putative hexose transporters deduced from the genome sequence of *L. bicolor* (Basidiomycota, ectomycorrhizal, Martin *et al*., 2008), *Coprinopsis cinerea*, (Basidiomycota, saprotroph, http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html), *Phanerochaete chrysosporium* (Basidiomycota, wood-decaying, Martinez *et al*., 2004), *Ustilago maydis* (Basidiomycota, plant pathogen, Kämper *et al*., 2006), *Cryptococcus neoformans* (Basidiomycota, human pathogen, Loftus *et al*., 2005) *Aspergillus niger* (Ascomycota, saprotroph, Pel *et al*., 2007), *Saccharomyces cerevisiae* (Ascomycota, saprotroph, Boles & Hollenberg, 1997), and *Arabidopsis thaliana* were compared with further selected fungal and human protein sequences using DIALIGN. Phylogenetic relationships were estimated by maximum-likelihood analysis with RAxML using the JTT model of amino acid substitution and additionally accounting for heterogeneous substitution rates. Numbers above branches denote bootstrap values from 200 replicates (only values higher than 50% shown). The tree was rooted with the *Arabidopsis* genes (see Supplementary material, Fig. S1). The phylogenetic tree was split into two parts for better legibility.

any carbon source for 1 wk. After medium exchange and addition of glucose (final concentration 10 mM), mycelia were cultivated for up to 16 d (without exchange of the respective growth medium) and samples were taken at different times. After DNA removal, first-strand cDNA was synthesized and expression analysis was performed by quantitative RT-PCR using gene-specific primers. To compare the expression levels of different members of the sugar transporter gene family, gene expression was calibrated to 10 000 molecules of 18S rRNA (Fig. 2).

As no transcripts were detectable for *Lacbi1:306961*, it can be supposed to be merely a pseudogene. The other genes can be grouped according to their maximum level of expression in substrate mycelium as follows: one to 10 mRNA molecules per 10 000 rRNAs (eight genes: *Lacbi1:380081* > *Lacbi1:183424* > *Lacbi1:304755*, *Lacbi1:142821* and *Lacbi1:385212* > *Lacbi1:305352*, *Lacbi1:301992*, *Lacbi1:191542*), 0.1 to 0.9 mRNA molecules per 10 000 rRNAs (two genes: *Lacbi1:313180* > *Lacbi1:298959*), and transcript abundances below 0.1 mRNA molecules per 10 000 rRNAs (four genes: *Lacbi1:297020* > *Lacbi1:314210* > *Lacbi1:300971* > *Lacbi1:397934*).

Three different gene expression patterns could be distinguished. Four genes (*Lacbi1:380081*, *Lacbi1:300971*, *Lacbi1:298959*, and *Lacbi1:314210*) showed either unchanged transcript abundances or fluctuations unrelated to fungal growth. Three genes (*Lacbi1:301992*, *Lacbi1:397934*, and *Lacbi1:385212*) revealed a temporally restricted induction of gene expression after glucose addition, lasting for approx. 2–8 h before declining to the initial level again (Fig. 2). Seven genes showed either a fast (*Lacbi1:305352*, *Lacbi1:313180*, *Lacbi1:191542*, *Lacbi1:297020*, and *Lacbi1:183424*) or slow (*Lacbi1:304755* and *Lacbi1:142821*) repression of their transcript abundance after sugar addition to carbon-starved fungal mycelia. A correlation between the expression pattern and the phylogenetic relationship of the proteins was not observed.

To investigate the effect of different carbon sources, mycelia were pre-cultivated in liquid culture in the absence of any carbon source for 1 wk before the addition of carbohydrates at final concentrations of 2 or 10 mM. After cultivation for an additional 2 d (with an exchange of the respective growth medium once a day) mycelia were collected and expression analysis was performed (Fig. 3).

In agreement with the previous experiment, the transcript abundances of most (10 out of 14) of the potential sugar transporter genes were reduced in the presence of glucose or fructose compared with carbohydrate starvation. For most of these genes, a weaker repression was observed at a lower glucose concentration. The only exception was *Lacbi1:397934*, which revealed a more pronounced reduction in gene expression in the presence of 2 mM instead of 10 mM glucose. Compared with glucose, the impact of fructose on gene expression was weaker (exceptions are *Lacbi1:301992*, *Lacbi1:380081* and *Lacbi1:297020*).

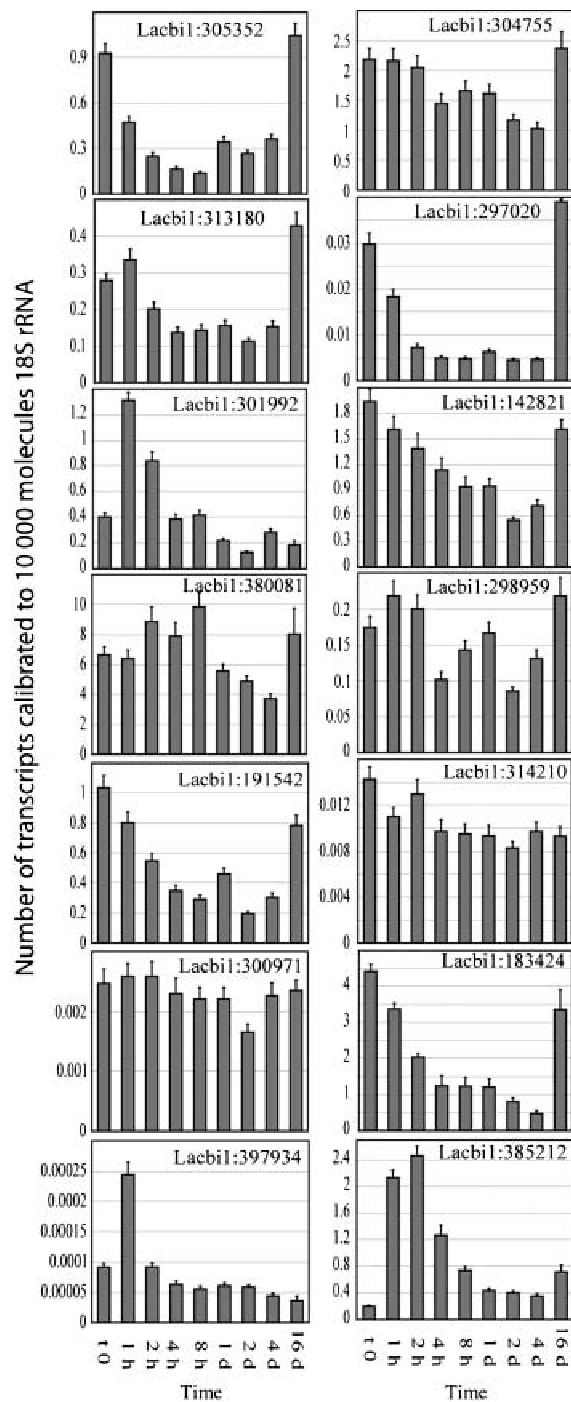


**Fig. 2** Impact of carbon starvation on the expression of members of the sugar porter gene family in *Laccaria* hyphae. *L. bicolor* mycelia were pre-cultivated in liquid culture in the absence of any carbon source for 1 wk. After medium exchange and addition of glucose (final concentration 10 mM), mycelia were further cultivated for up to 16 d (without a change of the respective growth medium) and mycelial samples were taken at different times. Total RNA was isolated and first-strand cDNA was synthesized after DNA removal. Expression analysis was performed by quantitative RT-PCR using gene-specific primers and was calibrated to 10 000 molecules of 18S rRNA.

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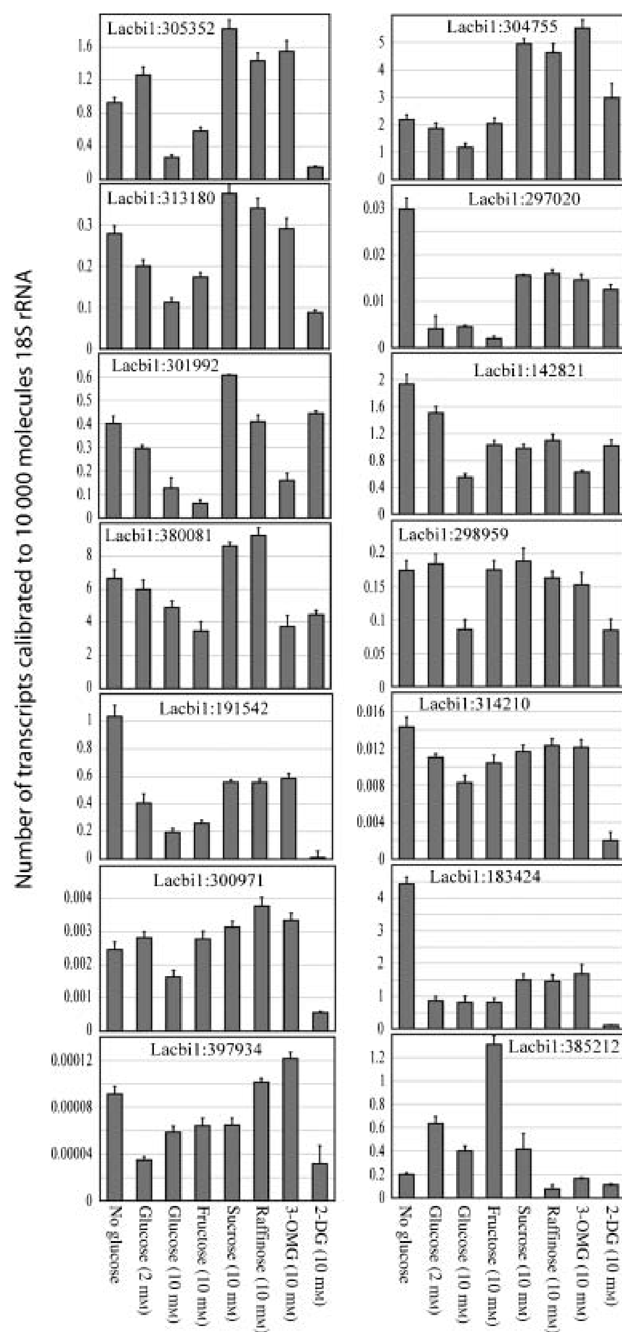


**Fig. 3** Impact of carbon sources on the expression of members of the *Laccaria* sugar porter gene family. *L. bicolor* mycelia were pre-cultivated in liquid culture in the absence of any carbon source for 1 wk. After medium exchange and addition of different carbon sources (final concentration 2 and 10 mM, respectively), mycelia were cultivated for a further 2 d (with a change of the respective growth medium once a day). Expression analysis was performed by quantitative RT-PCR using gene-specific primers and was calibrated to 10 000 molecules of 18S rRNA.

*Lacbi1:385212* revealed an enhanced expression in the presence of hexoses, with the strongest effect in the presence of fructose, while glucose analogs (3-O-methyl glucose (3-OMG), 2-D-glucose) did not affect the transcript abundance.

For the majority of the genes (10 out of 14) the presence of disaccharides (sucrose or raffinose) had only a minor impact on their transcript abundances. Exceptions are *Lacbi1:191542*, *Lacbi1: 297020*, and *Lacbi1:183424*, where gene expression was reduced compared with carbohydrate starvation, and *Lacbi1:304755*, which revealed elevated transcript abundance.

## Impact of nitrogen nutrition on sugar transporter gene expression

Fungal carbohydrate and nitrogen nutrition are interconnected and thus affect each other at the regulatory level (Baars *et al.*, 1995; Nehls, 2004). To look at the impact of nitrogen nutrition on sugar transporter gene expression, the presence of four different nitrogen sources found in forest soils (nitrate, ammonium, urea, amino acids) was compared with nitrogen depletion. Eight out of 14 genes showed no or minor changes in their transcript abundances, while four genes revealed a tendency towards a mild gene repression in the presence of any nitrogen source (data not shown). These data thus indicated only a minor impact of nitrogen nutrition on sugar transporter gene expression in *L. bicolor*.

## Gene expression and ectomycorrhiza formation

Six genes (*Lacbi1:305352*, *Lacbi1:313180*, *Lacbi1:301992*, *Lacbi1:191542*, *Lacbi1:304755* and *Lacbi1:385212*) showed a strongly enhanced transcript abundance upon ectomycorrhiza formation when compared with the extraradical mycelium (Fig. 4). For four of them (all revealing transcript abundances above 1 mRNA molecule per 10 000 rRNA molecules in mycorrhizas), gene expression was highest in ectomycorrhizas compared with all other investigated conditions. The expression of a further five genes (*Lacbi1:380081*, *Lacbi1:300971*, *Lacbi1:297020*, *Lacbi1:142821* and *Lacbi1:298959*) was either slightly increased or not affected in ectomycorrhizas compared with the extraradical mycelium. Only two genes (*Lacbi1:397934* and *Lacbi1:314210*) revealed a significant ($P < 0.0005$), and one gene (*Lacbi1:183424*) a tendentious, reduction in their transcript abundances upon ectomycorrhiza formation. However, since only one of these genes (*Lacbi1: 183424*) was expressed at a higher level (above 1 mRNA molecule per 10 000 rRNA molecules), ectomycorrhizas revealed an overall strongly enhanced expression level of putative hexose transporter genes compared with hyphae of the extraradical mycelium.

When compared with mycelia grown in submerse culture (that are well supported with carbohydrates in the growth medium) the extraradical mycelium (supported with carbohydrates by ectomycorrhizas by long-distance transport) revealed a lower

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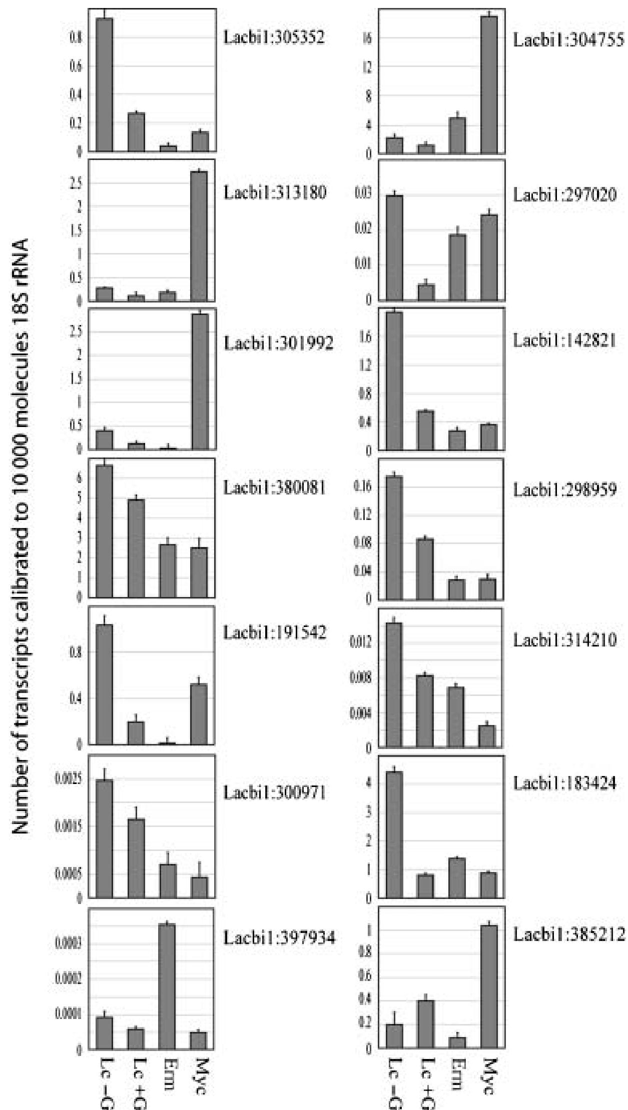


**Fig. 4** Expression of members of the sugar porter gene family upon ectomycorrhiza formation. Total RNA was isolated from *L. bicolor* mycelia grown for 2 d in liquid culture with 10 mM glucose (Lc +G) or the absence of any carbon source (Lc –G), the extraradical mycelium (ERM), and ectomycorrhizas (Myc) obtained from inoculated poplar plants in a Petri dish system according to Hampp *et al*. (1996). Expression analysis was performed by quantitative RT-PCR using gene-specific primers and was calibrated to 10 000 molecules of 18S rRNA.

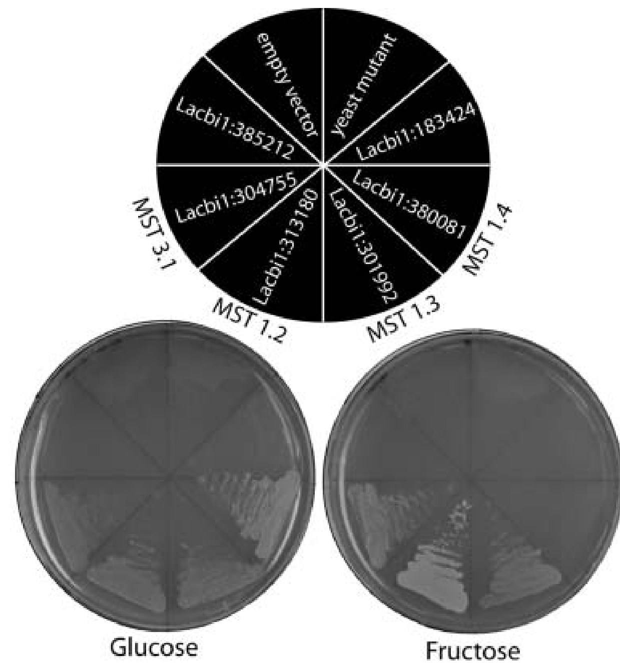


**Fig. 5** Functional complementation of a yeast mutant defective in hexose import by selected members of the *Laccaria* sugar porter gene family. Six *Laccaria* genes coding for potential hexose transporters (*Lacbi1:385212*, *Lacbi1:304755*, *Lacbi1:313180*, *Lacbi1:301992*, *Lacbi1:380081*, and *Lacbi1:183424*) were cloned into the yeast expression vector pDR196 (Rentsch *et al*., 1995) and transformed together with the empty vector into the yeast mutant EBY.VW4000 (Wieczorke *et al*., 1999) defective in hexose import. Single yeast transformants and the nontransformed strain were analyzed for their growth on supplemented YNB medium containing either glucose or fructose (2% final concentration) as sole carbohydrate source. *Laccaria* genes that were capable of complementing the hexose import defect of the yeast mutant were renamed according to their phylogenetic affiliation.

transcript abundance for eight of the genes. For four genes the expression rate was higher in extraradical hyphae, indicating a different regulatory effect of externally and internally offered carbohydrates on *L. bicolor* sugar transporter gene expression. This is in contrast to data observed for *A. muscaria* (Nehls *et al*., 2007), where both identified sugar transporter genes (that were obtained from mycorrhizas) revealed similar expression profiles in hyphae grown in submerse culture in the presence of external sugars or those obtained from functional ectomycorrhizas.

## Transport properties of selected members of the *Laccaria* sugar transporter gene family

All potential hexose transporter genes revealing a mycorrhiza-regulated induction of gene expression compared with the extraradical mycelium (*Lacbi1:304755*, *Lacbi1:313180*, *Lacbi1:301992*, and *Lacbi1:385212*) and transcript abundance above one mRNA molecule per 10 000 rRNAs in ectomycorrhizas were investigated for their hexose transport properties by heterologous expression in yeast. Additionally, two highly expressed but not mycorrhiza-induced genes were included in this analysis (*Lacbi1:380081*, *Lacbi1:183424*). As these six genes represent the most abundantly expressed members of the SP gene family, the potential sugar uptake capacity of *L. bicolor* hyphae in ectomycorrhizas could be estimated. Out of the six investigated genes, four (*Lacbi1:304755*, *Lacbi1:313180*, *Lacbi1:301992*, *Lacbi1:380081*) were capable of restoring the growth defect of the yeast mutant (Fig. 5).

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After this proof of function of the corresponding proteins, these genes were renamed as *LbMST1.2* (*Lacbi1:313180*), *LbMST1.3* (*Lacbi1:301992*), *LbMST1.4* (*Lacbi1:380081*), and *LbMST3.1* (*Lacbi1:304755*). To determine the import properties of the proteins, import studies with $^{14}$C-labeled glucose were performed. Three of the transporter proteins revealed similarly low $K_M$ values for glucose uptake (LbMST1.2, 58.6 (± 2.2) µM; LbMST1.3, 64.2 (± 7.5) µM; and LbMST3.1, 64.7 (± 6.6) µM). The corresponding genes were all induced upon ectomycorrhiza formation. The $K_M$ value of the fourth MST protein (that was not induced in ectomycorrhizas) was about seven times higher (LbMST1.4: 430.8 (± 31.9) µM).

To compare the transport properties of the proteins for glucose and other sugars, competition experiments were performed. Transgenic yeasts expressing the respective proteins were inoculated with radioactive-labeled glucose (final concentration close to the $K_M$ value) and a 15-fold excess of a competitor sugar. The ability of the competitor to inhibit the uptake of labeled glucose is shown in Fig. 6. The uptake rate of radioactive glucose in the absence of any competitor sugar was always set to 100%.

No inhibition of glucose uptake was observed for D-arabinose, indicating that the pentose is not imported by any of the investigated proteins. The presence of nonradioactive glucose inhibited the uptake of radioactive glucose by all investigated hexose transporters by approx. 85%. Glucose was always the best inhibitor. Surprisingly, although fructose as the sole carbon source conferred yeast growth in the presence of LbMST1.2, LbMST1.3, or LbMST3.1 (Fig. 5), a relative strong inhibitory effect on glucose uptake was only observed for LbMST1.2 (Fig. 6). For this protein the $K_M$ value for fructose was determined as 1108 (± 71) µM (data not shown), *c.* 17.6 times higher than its $K_M$ value for glucose. Because the inhibitory effects of fructose on glucose uptake were much smaller for LbMST1.3 and LbMST3.1, even larger differences in their respective $K_M$ values for both hexoses can be supposed. In agreement with the inability of LbMST1.4 to restore yeast growth on fructose as the sole carbohydrate source (Fig. 5), no fructose inhibition of glucose uptake was observed (Fig. 6).

Stereoisomers and analogs of glucose clearly affected the hexose uptake. Mannose and 2-deoxyglucose, but not glucosamine (all with a modified C-2 position compared with glucose), revealed significant ($P < 0.06$) inhibitory effects on glucose uptake. Comparably strong inhibitory effects as observed with unlabeled glucose were obtained for LbMST1.2 with mannose and 2-deoxyglucose, for LbMST1.3 with 2-deoxyglucose, and for LbMST3.1 with mannose. The glucose analog 3-OMG containing a modified C-3 position revealed some inhibitory effects on glucose uptake by all transporters investigated. However, effects as strong as those observed for glucose were obtained only for LbMST3.1. The C-4 position (galactose) seems to be more critical than C-2 or C-3. The stereoisomer galactose did not inhibit glucose uptake at all by LbMST1.3 and LbMST1.4. Only LbMST3.1 showed a strong inhibitory effect of galactose (comparable to that of unlabeled glucose), while LbMST1.2 revealed only a weak inhibition.

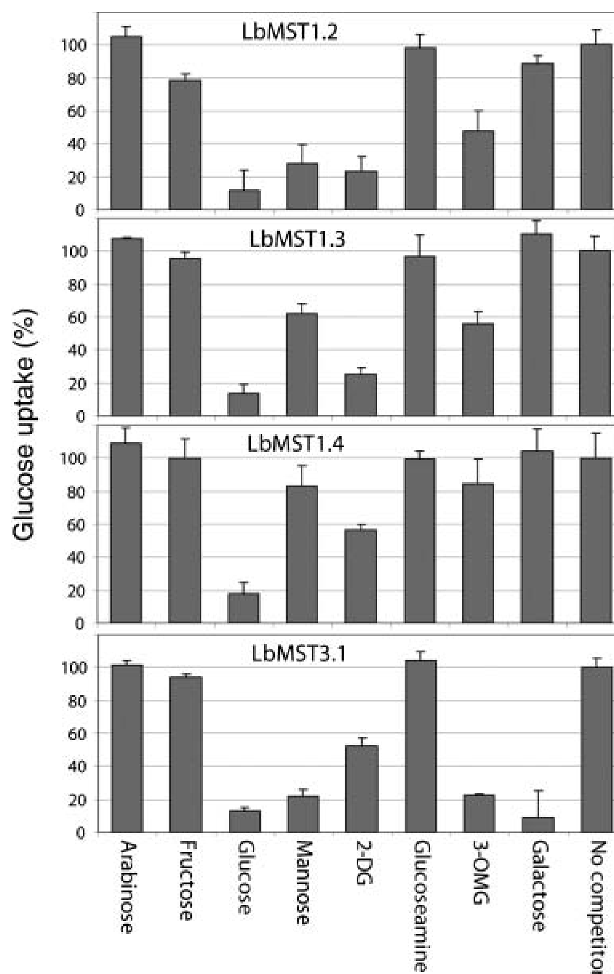


**Fig. 6** Substrate specificity of selected members of the *Laccaria* sugar porter gene family. *Laccaria* genes, capable of complementing the defect in hexose import of the yeast mutant EBY.VW4000 (Wieczorke *et al*., 1999), were investigated for their substrate spectrum by competition assays. Transgenic yeasts expressing the respective proteins were inoculated with radioactive-labeled glucose (final concentration around the respective $K_M$ value) and a 15-fold excess of a competitor sugar. The uptake rates of radioactive glucose are reported in percentage of uptake rates by a control without competitor added.

## Hexose uptake properties of *L. bicolor* hyphae

To compare the import properties of hexose transporters as characterized in yeast with that of *L. bicolor* hyphae, submerse cultures of *L. bicolor* were pre-cultivated in the absence of any carbon source for 1 wk. After medium exchange and addition of identical amounts of glucose and fructose (final concentration 4 mM each), the hexose content in the growth medium was followed over time (Fig. 7). Similar to hyphae of the EM

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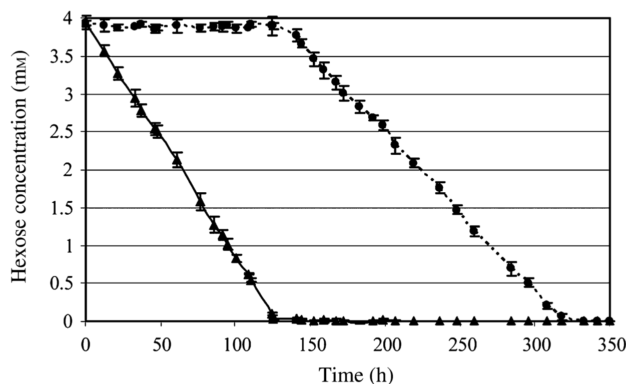


**Fig. 7** Hexose uptake by *Laccaria bicolor* mycelia grown in submerse culture. Mycelia were pre-grown in liquid Modified Melin Norkrans without any carbon source for 1 wk. After medium exchange, equimolar concentrations (4 mM each) of glucose (triangles) and fructose (circles) were added and the sugar content in the growth medium was followed for up to 2 wk.

fungus *A. muscaria*, *L. bicolor* consumed glucose first. However, unlike *A. muscaria*, *L. bicolor* hyphae did not consume large amounts of fructose until the glucose concentration was below the detection limit. This observation is in agreement with the large difference in $K_M$ values for glucose and fructose for the investigated hexose importer proteins. The maximal rates for consumption of glucose and fructose were similar (*c.* 5 µmol glucose $h^{-1}$ $g^{-1}$ FW and 3.4 µmol fructose $h^{-1}$ $g^{-1}$ FW), indicating comparable $V_{MAX}$ values of the respective hexose importers for both sugars.

## Discussion

Ectomycorrhizal fungal colonies are composed of several different hyphal networks with distinct functions that remain functionally interconnected (Cairney *et al.*, 1991): soil-growing hyphae for nutrient exploration; fungal strands or rhizomorphs for long-distance transport between different parts of the fungal colony; the fungal sheath of ectomycorrhizas, which serves as an intermediate storage for nutrients and metabolites that are exchanged between mycorrhizas and soil-growing hyphae/fruiting bodies; the Hartig net of ectomycorrhizas, where nutrients and metabolites are exchanged between plant and fungus; and fruiting bodies (containing themselves different hyphal networks). The large number of different hyphal functions might indicate a demand for adapted hexose uptake.

### Phylogeny and protein function

With 15 potential members, the genome of the EM model fungus *L. bicolor* contains a number of sugar transporter genes, which is comparable to that of other basidiomycetous (*Coprinopsis cinerea*, *Phanerochaete chrysosporium*, *Ustilago maydis*) or ascomycetous (*Aspergillus niger*, *Saccharomyces cerevisiae*) model fungi. Only the basidiomycetous human pathogen *Cryptococcus neoformans* showed a significant larger number with 48 predicted potential sugar transporter genes.

Members of the *Laccaria* SP gene family are found in different branches of our phylogenetic tree. Four *L. bicolor* members, of which three were proven to be functional by heterologous expression in yeast (this study), cluster together with 19 out of 20 *Saccharomyces* SP proteins (Boles & Hollenberg, 1997) and both so far functionally characterized EM fungal sugar transporters (Nehls *et al.*, 1998; Polidori *et al.*, 2007). Although phylogenetically closely related, the respective *L. bicolor* genes can be grouped by their expression behavior and their transport properties. *Lacbi1:313180/LbMST1.2* and *Lacbi1:301992/LbMST1.3* were ectomycorrhiza-induced and both respective proteins turned out to be high-efficiency glucose importers ($K_M$ values, 58.6 and 64.2 µM, respectively). By contrast, *Lacbi1:305352* was mainly expressed under carbon starvation. Also *Lacbi1:380081/LbMST1.4* revealed its highest transcript abundance under these conditions but was, however, also strongly expressed in the extraradical mycelium and ectomycorrhizas. Together with its much lower affinity for glucose ($K_M$, 430.8 µM), the respective protein can thus be supposed to perform low-affinity but high-capacity basal glucose uptake of *L. bicolor* hyphae.

Even when *Lacbi1:304755/LbMST3.1* is phylogenetically less closely related to *LbMST1.2* and *LbMST1.3*, the expression profiles of the respective genes and the sugar transport properties of the proteins are similar. The gene clusters together with the second identified potential sugar transporter from the EM fungus *A. muscaria* for which no functional proof is yet available.

While yeast hexose transporters and all hexose transporters from EM fungi characterized to date have a clear preference for glucose uptake, BcFRT1 from *Botrytis cinerea* (Doehlemann *et al.*, 2005) is clearly a fructose importer. *Lacbi1:385212* (this study) clusters together with *BcFRT1* and its expression was (similar to the gene from *B. cinerea*) strongly increased in the presence of fructose. However, when heterologously expressed in yeast, Lacbi1:385212 revealed no hexose uptake capability.

Taken together, proven sugar importer function could be found in different branches of the SP gene family and no correlation between position in the phylogenetic tree and protein function could be drawn. However, for the majority of the members of this gene family, no functional characterization has yet been carried out, or functional analysis by heterologous expression in yeast has failed. The purpose of the respective proteins therefore remains unclear. The inability of genes to complement a yeast defect after heterologous expression, however, does not necessarily indicate another function of the deduced protein. Technical problems (e.g. instability of mRNA in yeast or mistargeting) or incorrect gene annotation might interfere with a successful complementation. Furthermore, only hexose transporter activity in the plasma membrane

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is investigated by this approach and members of the SP gene family might be localized in other membranes (e.g. endoplasmatic reticulum or the vacuole). Thus, subcellular localization has to be performed in future to clarify the potential function of the respective proteins.

## Carbohydrate uptake capacity of hyphae under carbon starvation

Compared with root exudates, sugars are rare in soils of forest ecosystems (Wainwright, 1993), thus limiting microbial propagation (Jonasson *et al.*, 1996a). Therefore, hexose uptake by soil-growing hyphae of EM fungi is assumed to be important for two reasons: additional carbohydrate nutrition for the EM fungal colony, and to reduce the competition for nutrient uptake by other soil microorganisms (Jonasson *et al.*, 1996a,b; Hogberg *et al.*, 2003; for reviews, see Cairney & Meharg, 2002). A further function of hexose importers might be avoidance of carbohydrate leakage by sugar reimport. Because sugars are present in fungal hyphae in large amounts compared with forest soils and are able to permeate the plasma membrane in a concentration-dependent manner (for a review, see Burgstaller, 1997), carbohydrate loss might be a constant problem, especially under conditions of carbon starvation.

Compared with well-carbohydrate-supported hyphae, the expression of 11 putative sugar transporter genes (including three with proven function of their deduced proteins) was either elevated (seven genes) or unchanged (four genes) when *L. bicolor* mycelia were grown in liquid culture under carbohydrate starvation. Five of these genes (*Lacbi1:305352*, *Lacbi:301997*, *Lacbi1:191542*, *Lacbi1:142821*, and *Lacbi1:183424*) revealed a high transcript abundance (at least 0.9 mRNA molecules per 10 000 rRNAs) and showed their highest expression levels under conditions of carbon starvation. Our data thus support, on a genome-wide level, the observation of Polidori *et al.* (2007), based on a single hexose transporter gene from the ascomycetous EM fungus *Tuber borchii*, indicating a strong demand of fungal hyphae for sugar uptake capacity under carbon limitation/starvation.

In yeast, sugar-dependent gene repression (as observed for seven of the *Laccaria* sugar transporter genes) is regulated in a hexokinase-dependent manner (Hohmann *et al.*, 1999). However, according to our data, only two of the affected *L. bicolor* hexose importer genes (*Lacbi1: 305352* and *Lacbi1: 313180*) could be assumed to be regulated by hexokinase-mediated catabolite repression. Here, presence of the glucose analog 3-OMG (which is imported but not phosphorylated by hexokinase) resulted in strong gene expression (as under carbon starvation), while the presence of 2-deoxyglucose (which is imported and phosphorylated by hexokinase) resulted in a similar reduction in transcript abundance to that caused by the presence of glucose. However, for the observed sugar-dependent repression of most investigated *L. bicolor* genes, other mechanisms must be supposed.

## Hexose uptake and ectomycorrhiza formation

Large amounts of the carbohydrates that are consumed by soil-growing hyphae originate from ectomycorrhizas, where hexoses are taken up efficiently at the plant–fungus interface. Out of the nine most strongly expressed genes of the *L. bicolor* SP gene family, only one (*Lacbi1: 314197*, which showed no hexose import activity when expressed in yeast) revealed a reduced (twofold) transcript abundance in mycorrhizas compared with the extraradical mycelium. Of the remaining eight genes (including all four genes with proven hexose import function of their corresponding proteins), six showed a three- to 25-fold higher expression in ectomycorrhizas, indicating a strongly increased sugar uptake capacity of *L. bicolor* hyphae in symbiosis compared with those of the extraradical mycelium. This genome-wide analysis of gene expression combined with functional analysis of selected hexose importers of *L. bicolor* therefore supports results from *A. muscaria* obtained previously with a very limited dataset (Nehls *et al.*, 1998; Nehls, 2004).

## Regulation of enhanced sugar transporter gene expression in ectomycorrhizas

Although both *A. muscaria* and *L. bicolor* strongly enhance their hexose uptake capacity in symbiosis, the underlying regulatory mechanisms are different.

An enhanced gene expression, as observed in functional ectomycorrhizas, can be mimicked by exposure of hyphae grown in liquid culture to elevated external hexose (glucose or fructose) concentrations in *A. muscaria.* Furthermore, both already identified hexose importer genes (*AmMST1* and *AmMST2*) revealed a lag phase of approx. 1 d before elevated transcript abundances were observed, and gene expression remained high as long as the external hexose concentration was above the $K_M$ values of the corresponding proteins. Lag phase and long-lasting enhanced gene expression were interpreted as an adaptation of EM fungal hyphae to a constant sugar supply, which is observed only at the plant–fungus interface under natural conditions and indicates the apoplastic hexose concentration as a regulator of hexose importer gene expression in symbiosis (for a current review, see Nehls *et al.*, 2007).

In *L. bicolor*, only two (*LbMST1.3*/*Lacbi1:301992* and *Lacbi1:385212*) out of the six sugar transporter genes with a mycorrhiza-dependent elevated transcript abundance revealed an induced gene expression upon hyphal exposure to elevated external hexose concentrations. Furthermore, sugar enhanced gene expression of these genes was only short-lived (*c.* 8 h in maximum) and the induction was only half of that observed in ectomycorrhizas in the case of *LbMST1.3*/*Lacbi1:301992*, contrasting with the results obtained (with a limited dataset) for *A. muscaria*. The remaining four genes showed either no effect (*LbMST1.4*/*Lacbi1:380081*) or an even reduced transcript abundance (*LbMST1.1*/*Lacbi1:305352*, *LbMST1.2*/*Lacbi1:313180*, *LbMST3.1*/*Lacbi1:304755*) when hyphae

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were grown in liquid culture at elevated external hexose concentrations. Together, these data clearly indicate that the observed enhanced gene expression in *L. bicolor* ectomycorrhizas is not regulated by differences in the apoplastic hexose concentration, as in the case of *A. muscaria*, but may be controlled by the developmental process of ectomycorrhiza formation. However, also in *A. muscaria*, a developmental control of genes involved in sugar metabolism (trehalose biosynthesis) has been shown (Fajardo López *et al.*, 2007), demonstrating that the physiological adaptation of fungal hyphae is controlled by different regulatory mechanisms in symbiosis.

### Sugar consumption by EM fungal hyphae

A further distinct difference between *A. muscaria* and *L. bicolor* was the behavior of carbon-starved mycelia after glucose addition. While no lag phase for glucose uptake was observed for *L. bicolor* (this study), *A. muscaria* hyphae (Wiese *et al.*, 2000), and also those of *Hebeloma cylindrosporum* (Salzer & Hager, 1991), needed *c.* 1 d before glucose import was maximal. The reason for this immediate glucose import by *L. bicolor* mycelia after glucose addition is presumably that seven out of the nine most strongly expressed sugar transporter genes revealed a high transcript abundance under carbon starvation, while only two genes were sugar-induced. By contrast, both already characterized *A. muscaria* hexose transporter genes (*AmMST1* and *AmMST2*) needed *c.* 1 d of sugar exposure before their expression increased (Nehls *et al.*, 1998; Nehls, 2004), a lag phase identical to that observed for glucose uptake by *A. muscaria* hyphae.

Furthermore, the maximal hexose uptake rate of hyphae grown in submerse culture differed for *A. muscaria* and *L. bicolor*. *A. muscaria* took up glucose with a maximal rate of 34.6 μmol $h^{-1}$ $g^{-1}$ FW and fructose with a maximal rate of 21.2 μmol $h^{-1}$ $g^{-1}$ FW (Wiese *et al.*, 2000). By contrast, the maximal uptake rate for *L. bicolor* hyphae was much lower (6.9 times for glucose and 6.3 times for fructose). However, taking into account the fact that the expression of *AmMST1* and *AmMST2* is about a factor of six lower in carbohydrate-starved *A. muscaria* mycelia than in hyphae exposed to elevated external hexose concentrations, the overall hexose consumption of *A. muscaria* and *L. bicolor* hyphae is presumably similar under conditions of carbohydrate starvation.

One explanation for the increased hexose import capacity of *A. muscaria* mycelia compared with those of *L. bicolor* could be the different hyphal glucose contents of both EM fungi when exposed to elevated external hexose concentrations. When *A. muscaria* hyphae are grown well supported with glucose in submerse culture, they contain *c.* 10–20 mg glucose $g^{-1}$ DW (Wallenda, 1996), while *L. bicolor* mycelia have a much lower glucose content when grown under comparable conditions (1 mg glucose $g^{-1}$ DW; Bois *et al.*, 2006). Increased hyphal glucose content together with a modified carbon metabolism, however, may result in an enhanced carbohydrate loss by leakage over the plasma membrane. As a consequence, a higher hexose import capacity of the fungus (*A. muscaria*) would be needed for compensation.

### Fructose discrimination by *L. bicolor* hyphae and its consequence at the plant–fungus interface

Submerse cultures of *A. muscaria* (Hampp *et al.*, 1995; Wiese *et al.*, 2000), *Hebeloma cylindrosporum* (Salzer & Hager, 1991), *Coenococcum geophilum* (Stülten, 1996), and *L. bicolor* mycelia preferentially took up glucose from a 1 : 1 mixture of glucose and fructose. However, unlike *A. muscaria* or *H. cylindrosporum* hyphae, which imported fructose (with lower efficiency) parallel to glucose, *L. bicolor* did not visibly reduce the fructose content in the growth medium unless it became glucose-depleted. As carbon nutrition of the fungal partner in ectomycorrhizas is supposed to be based on apoplastic hydrolysis of plant-derived sucrose, *L. bicolor* hyphae may take up mainly glucose and lose a large portion of the remaining fructose. Compared with other fungi (e.g. *A. muscaria* or *H. cylindrosporum*) this behavior may result in less efficient carbohydrate exploitation by *L. bicolor* hyphae in symbiosis. As *H. cylindrosporum* and *L. bicolor* are phylogenetically more closely related than *A. muscaria* and *H. cylindrosporum* (Garnica *et al.*, 2007), this inefficient fructose uptake behavior of *L. bicolor* could be supposed as the exception rather than the rule.

### Conclusion

Based on the low sugar content in forest soils that could be a growth-limiting factor in these ecosystems, the genome wide analysis of the *L. bicolor* SP gene family indicates two potential functions of sugar importers in EM fungi, initially postulated on the basis of results with single hexose transporters from *A. muscaria* (Nehls *et al.*, 1998; Nehls, 2004) and *T. borchii* (Polidori *et al.*, 2007): sugar uptake by soil-growing hyphae for improved carbon nutrition and a reduction of nutrient competition by other soil microorganisms; and generation of a strong carbohydrate sink at the plant–fungus interface in symbiosis. By contrast with the situation in *A. muscaria*, the strongly enhanced hexose uptake capacity of mycorrhizal hyphae is not regulated in a sugar-dependent manner in *L. bicolor*. Instead, developmental regulation can be supposed.

## Acknowledgements

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## Supplementary Material

The following supplementary material is available for this article online:

**Fig. S1** Phylogenetic relationships of the deduced protein sequences of *the Laccaria* sugar porter gene family with known fungal hexose transporters.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2008.02539.x
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