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.


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 (Blassius 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 & Högborg, 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., 2000; Wright et al., 2000).

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 Petri dishes or in liquid culture for up to 16 d with MMN (Modified Melin Norkrans; Marx, 1969) medium in

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.

Expression analysis 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 realtime PCR system (BioRad, Hercules, CA, USA). Specific primers for L. bicolor 18S RNA 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/Lach1/Lach1.home.html) are as follows: 18S rRNA, 5′-CAGAGCCAGCGATTTCCTTTC-3′ and 5′-GTCTTGGGCTCCCACAAAC-3′; Lach1:313180, 5′-GAACTTTTGGAATCGCTTATG-3′ and 5′-TGACAGCAAGACATGTAG-3′; Lach1:305352, 5′-GTCATCTTCCACTGCGAG5′-3′ and 5′-AGAAGACTTTGCGCTCAAG-3′; Lach1:380081, 5′-CGTCGTCACACTCGTGATAG-3′ and 5′-GATGAAACTCGCGAAACAC-3′; Lach1:301992, 5′-TGCGTGCTATCTCGATGTG-3′ and 5′-GATTGAACTGATCGAGG-3′; Lach1:314210, 5′-AGTGCACTCC-AATGGGCTC-3′ and 5′-GCCATGCTGGCTGATTTG-3′; Lach1:298959, 5′-AAGTTACCGCCCAAATG-3′ and 5′-TTGTTTTTGTGTTGATAAAGG-3′; Lach1:183424, 5′-TTAACATCCTCGCAATG-3′ and 5′-TGAGCTTTGACGTTCCTCGT-3′; Lach1:306961, 5′-GACTCCCTGACTCCTACAGAAG-3′ and 5′-ACACAGCTGCATCTCTTC-3′; Lach1:300971, 5′-ATTCCCTTCCTGCTGTCCTC-3′ and 5′-TGTCACTTCCTCCACAAACC-3′; Lach1:397934, 5′-GGGCTATTACCTTCCTTTCTTC-3′ and 5′-AACACCTCCTCCACGCAAGC-3′; Lach1:191542, 5′-GGCGCTTCC-TACCCTGAAAC-3′ and 5′-ACCTGTATCAGCCTCTCTTC-3′; Lach1:305045, 5′-TTTGGGAAATCTACACTTTCGC-3′ and 5′-ATGTTGTGTCCTCCTGTTG-3′; Lach1:142821, 5′-GGCAAGTATCGCTCCTGC-3′ and 5′-GATTGCAACTGCTCATTG-3′; Lach1:297020, 5′-AACACAGCCCATACCTCC-3′ and 5′-GAGGTTATATGCGAGC-3′; Lach1:385212, 5′-GAGGTTATACGTCTTCCGAG3′ and 5′-TGTGTTCTCCTCTGCGGAAC-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 gene family members was investigated over a range of five orders
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 RNA 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, © The Authors (2008). Journal compilation © 2008). 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 µmol$^{-1}$). 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 (strain S238N-H82) genome (Martin et al., 2008), as predicted by the JGI (available at http://genome.jgi-psf.org/Labi1/Labi1.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
importers with proven hexose transport capabilities (all S. cerevisiae transporters, Wiczkorte et al., 1999; AmMST1, 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, PI. 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/Lacb1/Lacb1.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; Wiczkorte et al., 1999; Doehlemann et al., 2005; Polidori et al., 2007) as a template. Similar gene numbers as in L. bicolor 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 (Lacb1:304755, Lacb1:313180, Lacb1:301992, Lacb1:385212, Lacb1:380081, Lacb1:183424), the corresponding cDNAs were amplified for functional analysis (see below). For one of these genes (Lacb1:301992), 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 (Supporting Information, 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: Lacb1:301992, Lacb1:380081; cluster 3: Lacb1:183424, Lacb1:314210) 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. cerevisiae (which clusters together with Lacb1:191542) was shown to be a glycerol transporter (Ferreira et al., 2005), while BcFRT1 from Botrytis cinerea (which clusters together with Lacb1:385212) 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
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 Supporting Information, 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*).
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 submers 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
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 submersed culture in the presence of external sugars or those obtained from functional ectomycorrhizas.

**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.

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, *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.

**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.
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 14C-labeled glucose were performed. Three of the transporter proteins revealed similarly low $K_M$ values for glucose uptake (LbMST1.2, 58.6 ($\pm$ 2.2) $\mu$M; LbMST1.3, 64.2 ($\pm$ 7.5) $\mu$M; and LbMST3.1, 64.7 ($\pm$ 6.6) $\mu$M). 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 ($\pm$ 31.9) $\mu$M).

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 ($\pm$ 71) $\mu$M (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
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/fruited bodies; the Hartig net of ectomycorrhizas, where nutrients and metabolites are exchanged between plant and fungus; and fruited 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. *Lachi*:313180/LbMST1.2 and *Lachi*: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, *Lachi*:305352 was mainly expressed under carbon starvation. Also *Lachi*: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 *Lachi*: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* (Doehlemman et al., 2005) is clearly a fructose importer. *Lachi*: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, *Lachi*: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...
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 (Lachi:305352, Lachi:301997, Lachi:191542, Lachi:142821, and Lachi:183424) revealed a high transcript abundance (at least 0.9 mRNA molecules per 10 000 RNAs) and showed their highest expression levels under conditions of carbon starvation. Our data thus support, on a genome-wide level, the observation of Polidor 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 (Lachi:305352 and Lachi: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 (Lachi: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/Lachi:301992 and Lachi: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/Lachi:301992, contrasting with the results obtained (with a limited dataset) for A. muscaria. The remaining four genes showed either no effect (LbMST1.4/Lachi:380081) or an even reduced transcript abundance (LbMST1.1/Lachi:305352, LbMST1.2/Lachi:313180, LbMST3.1/Lachi:304755) when hyphae...
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* (Salzet & 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 submers 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 submersere 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

Submersic cultures of *A. muscaria* (Hampp et al., 1995; Wiese et al., 2000), *Hebeloma cylindrosporum* (Salzet & Hager, 1991), *Coenococcus 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.

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References


**Supporting Information**

Additional supporting information may be found in the online version of this article.

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

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