Carbon Allocation in Ectomycorrhizas: Identification and Expression Analysis of an *Amanita muscaria* Monosaccharide Transporter

Uwe Nehls, Joachim Wiese, Martin Guttenberger, Rüdiger Hampp

Universität Tübingen, Physiologische Ökologie der Pflanzen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

Accepted 1 December 1997.

Ectomycorrhizas are formed between certain soil fungi and fine roots of predominantly woody plants. An important feature of this symbiosis is the supply of plant-derived carbohydrates to the fungus. As a first step toward a better understanding of the molecular basis of this process, we cloned a monosaccharide transporter from the ectomycorrhizal fungus *Amanita muscaria*. Degenerate oligonucleotide primers were designed to match conserved regions from known fungal sugar transporters. A cDNA fragment of the transporter was obtained from mycorrhizal mRNA by reverse transcription–polymerase chain reaction. This fragment was used to identify a clone (AmMst1) encoding the entire monosaccharide transporter in a *Picea abies*/*A. muscaria* mycorrhizal cDNA library. The cDNA codes for an open reading frame of 520 amino acids, showing best homology to a *Neurospora crassa* monosaccharide transporter. The function of AmMST1 as monosaccharide transporter was confirmed by heterologous expression of the cDNA in a *Schizosaccharomyces pombe* mutant lacking a monosaccharide uptake system. AmMst1 was constitutively expressed in fungal hyphae under all growth conditions. Nevertheless, in mycorrhizas as well as in hyphae grown at monosaccharide concentrations above 5 mM, the amount of AmMst1 transcript increased fourfold. We therefore suggest that AmMst1 is upregulated in ectomycorrhizas by a monosaccharide-controlled mechanism.

Ectomycorrhizas are symbiotic associations of fine roots of higher plants, mostly trees, with certain soil fungi (usually ascomycetes and basidiomycetes). The dominant trees of temperate forests, members of the *Fagaceae* and the *Pinaceae*, are obligate ectomycorrhizal symbionts. In this symbiosis, neither the root nor the fungus function independently but form a unit with adapted metabolic pathways and controlled exchange of metabolites. It is a common notion that in this

type of symbiosis, mutual benefit between the partners is due to the exchange of plant-derived carbohydrates in exchange for amino acids and nutrients supplied by the fungus (Harley and Smith 1983; Smith and Read 1997). The first evidence for carbon transfer from the plant to the fungus was provided by Melin and Nilsson (1957). More recently, a number of studies have provided further information (for a review see Hampp and Schaeffer 1995; Schaeffer et al. 1995), but the nature of the transferred carbohydrates in mycorrhizas is still unknown.

Sucrose is the major transport form of photoassimilates in most higher plants and therefore it is of special interest for carbon transfer in organismic interactions. The growth of ectomycorrhizal fungi on sucrose as a carbon source is usually negligible or very poor (Salzer and Hager 1991). However, both glucose and fructose support growth of ectomycorrhizal fungi (Salzer and Hager 1991; Schaeffer et al. 1995). It is therefore assumed that sucrose is hydrolyzed to glucose and fructose by a plant-derived, cell-wall-bound, acidic invertase. In addition, there is evidence that monosaccharides are imported by fungal protoplasts via an ATP- and proton-potential-dependent mechanism (Chen and Hampp 1993).

In order to characterize this monosaccharide-uptake system in *Amanita muscaria*, we used a molecular approach. In this work, we describe the isolation of a cDNA encoding an *A. muscaria* monosaccharide transporter (AmMst1) and the expression pattern of the corresponding gene in ectomycorrhizas and mycelia grown under various conditions.

RESULTS

Isolation of cDNA clones coding for an *A. muscaria* monosaccharide transporter.

As a first step toward the cloning of genes for sugar transporters from *A. muscaria*, degenerate oligonucleotide primers were designed to match conserved regions from known sequences of fungal monosaccharide transporters (Fig. 1). With the exception of SNF3, RGT2, HXT9, and HXT14 of *Saccharomyces cerevisiae* and HGT1 of *Kluyveromyces lactis*, all investigated monosaccharide transporters have the consensus amino acid sequences. The primers were used for polymerase chain reaction (PCR) with a template consisting of single-stranded cDNA from reverse transcription of RNA from *Picea abies*/*A. muscaria* mycorrhizas. Amplified DNA fragments approximately 500 bp in length were subcloned into a plasmid

Corresponding author: Uwe Nehls, Universität Tübingen, Botanisches Institut, Physiologische Ökologie der Pflanzen, Auf der Morgenstelle 1, 72076 Tübingen, Germany; Telephone: +49 7071 2977657; Fax: +49 7071 296155; E-mail: uwe.nehls@uni-tuebingen.de

Nucleotide and/or amino acid sequence data have been submitted to the EMBL data base as accession number Z83828.

vector and 15 of the resulting clones were partially sequenced. All of the clones contained identical cDNA inserts, showing homology to fungal monosaccharide transporters. This cDNA fragment was further used for the isolation of cDNA clones from a *P. abies/A. muscaria* mycorrhizal cDNA library (U. Nehls, unpublished). Altogether, 200,000 clones were screened under high-stringency conditions, yielding seven independent clones with significant hybridization signals. One of these clones (1,520 bp in length) was used for a further screen of the mycorrhizal cDNA library. Three hundred and fifty thousand clones were screened under low-stringency conditions (35°C hybridization temperature, two washes at 35°C for 15 min each). Fifteen independent clones yielded significant hybridization signals. By partial sequencing, all clones were found to be identical at the nucleic acid level. Out of five clones with almost identical 5' ends, the longest cDNA clone (AmMst1, EMBL accession number Z83828) had a length of 1,711 bp and contained an open reading frame that could code for a protein of 520 amino acids with a molecular mass of 56,455 Da (Fig. 2). The deduced protein sequence of AmMST1 revealed 12 putative transmembrane domains, arranged in two groups of six each separated by a hydrophilic spacer of 65 amino acids. This is a pattern typical for the superfamily of transmembrane facilitators (Marger and Saier 1993). The best homology to the deduced protein was obtained for a *Neurospora crassa* monosaccharide transporter (Madi et al. 1997). A comparison of the deduced protein se-

quence of AmMST1 with other known fungal monosaccharide transporters is shown in Figure 3 and Table 1.

We assume that the AmMst1 cDNA encodes the entire transporter protein. The sequence upstream of the proposed initial start-ATG (38 bp) contains 2 stop codons within the reading frame (Fig. 2), which was carefully checked by repeated sequencing of this region. Furthermore, five of the known monosaccharide transporters, RCO3, SNF3, HGT1, HXT9, and STL1, code for proteins with N termini of similar length.

AmMst1 was found to be encoded by a single-copy gene in *A. muscaria* (Fig. 4). Digestion of genomic DNA with different restriction enzymes that do not cut within the AmMst1 cDNA (*EcoRI*, *SalI*, *KpnI*, *XbaI*, and *XhoI*) showed only single hybridization bands even under medium-stringency hybridization conditions (50°C hybridization temperature), using the entire AmMst1 cDNA as probe. This result ensures that only AmMst1 is recognized in Northern (RNA) blot analysis by the cDNA probe.

Heterologous expression of AmMst1.

In order to test whether the AmMST1 protein functions as monosaccharide transporter, the cDNA was cloned in sense and antisense orientation into a *Schizosaccharomyces pombe* expression vector (SAP-E; Truernit et al. 1996). The internal start ATG of the AmMst1 cDNA was used for expression in the sense construct. An *S. pombe* mutant lacking an import

Primer consensus	L	T
	FQQLTG	ETKGLS
AmMST1 <i>A. muscaria</i>	IQAW.....INF	CVP.....LEQ
RCO3 <i>N. crassa</i>	LQM.....VNF	MVY..SKI..LEQ
RGT2 <i>S. cerevisiae</i>	IQA...AS..INF	AVY..RG..LEE
SNF3 <i>S. cerevisiae</i>	LQA...FS..INF	TVY.....LEE
HXT4 <i>S. cerevisiae</i>	IQS.....DNY	FVP.....LEE
HXT7 <i>S. cerevisiae</i>	IQS.....DNY	VVP.....LEE
HXT6 <i>S. cerevisiae</i>	IQS.....DNY	VVP.....LEE
HXT11 <i>S. cerevisiae</i>	IQS.....DNY	FVP.....LEE
LGT3 <i>S. cerevisiae</i>	IQS.....DNY	FVP.....LEE
HXT3 <i>S. cerevisiae</i>	IQS.....DNY	FVP.....LEE
HXT1 <i>S. cerevisiae</i>	IQS.....DNY	FVP.....LEE
GAL2 <i>S. cerevisiae</i>	VQM.....NNY	FVP.....LEE
HXT10 <i>S. cerevisiae</i>	IQS.....CNY	FVC.....LEE
HXT5 <i>S. cerevisiae</i>	IQS.....DNY	FVP.....LEE
SUC2 <i>S. cerevisiae</i>	IQS.....DNY	FVP.....LEE
YNR072w <i>S. cerevisiae</i>	VQT...L..ENY	FL...I...LEE
HXT13 <i>S. cerevisiae</i>	VQT...L..ENY	FL...I...LEE
HXT2 <i>S. cerevisiae</i>	IQS.....NNY	FVC.....LEE
HXT8 <i>S. cerevisiae</i>	INS.....DNY	FVP.....LEE
permease <i>K. lactis</i>	IQS.....NNY	FVP.....LEE
RAG1 <i>K. lactis</i>	IQS.....DNY	FVP.....LEE
HXT14 <i>S. cerevisiae</i>	IMA....S..INY	LVP..RKKNEQE
HXT9 <i>S. cerevisiae</i>	IMA....S..INY	LVP..RKKNEQE
STL <i>S. cerevisiae</i>	TQF...FT..CNA	FYP..A..R..LEE
HGT1 <i>K. lactis</i>	AQMW...C..MNV	MFP....K..LEE

Fig. 1. Conserved amino acid stretches from known fungal monosaccharide transporters. A comparison of known fungal monosaccharide transporters revealed two highly conserved amino acid stretches, which were used for primer design. Their consensus sequence is shown on top. Amino acids identical to the consensus sequences are substituted by dots.

system for monosaccharides (Milbradt and Höfer 1994) was transformed with these constructs and with the expression vector alone. The transformants were grown in medium containing glucose as carbon source (Fig. 5). Only the yeast clone expressing AmMst1 cDNA in the sense orientation was able to grow on glucose, whereas both controls, the transformant containing AmMst1 in antisense orientation and the one containing the expression vector alone, did not grow. When cultivated on gluconate as a carbon source, the transformants and

the untransformed yeasts showed identical growth rates (data not shown), i.e., the different constructs had no influence on the viability of the yeast cells.

Expression analysis of the AmMst1 gene.

In nature glucose concentrations of 0.1 to 1 mM are usually found in soil (Wainwright 1993). Hyphae grown on agar plates as well as in liquid culture at monosaccharide concentrations of 1 mM constitutively expressed the AmMst1 gene.

```

1                               ATAACCTGCCCCCCCAAATTTTCTGGCCCTTGAACC
38 ATGCTCCAGCTGCTACGGGAATGGCGGCATAGATCCCCAAAAACAAATTTACTGGCATAGCCATGACAGCATTTC
   M P P A A T G N G G I D P K N K F T G I A M T A F 25
113 AGTGTCTTTTGGTGGTATCCTCTACGGTTACGACTGGCATTATCAGCGGTATCCTTGGCATGGACGATTTC AAG
   S A F G G I L Y G Y D T G I I S G I L A M D D F K 50
188 CGTACCTTTTGGTCAACAGGCTTCTGATGGCACATGTTCCCTTCCGCTCTCAAGCCAATCCCTGTTGTCTCTATT
   R T F G Q Q A S D G T C S L P L S S Q S L F V S I 75
263 CTCTCAGCTGGAACCTTCGTGCGAGCTCTCTTTGGTGTCTCCCATGGGTGACATTATCGGCAGAAAATGGGGTATC
   L S A G T F V G A L F G A P M G D I I G R K Y G I 100
338 GTCATAGCTGCTGCGATATTCTCGATCGGTATTGCCTTGCAAACAGCCTCAGTAACGGCAGCTCCCTTCGTGCTT
   V I A A A I F S I G I A L O T A S V T A A P F V V 125
413 GGTCGTGTCTTCGCCGGTCTTGGTGTCTGGTTTCTTGCTGCTGGTTCCTATGTATCAGTCCGAATGCTCACCA
   G R V F A G L G V G L V S C L V P M Y Q S E C S P 150
488 AAATGGATCCGCGGTGCCGTAGTATCATTGTACCAATGGGCCATTACGATTGGAATCTTGCTTGTCTGCCGTCATC
   K W I R G A V V S L Y Q Y A I T I G I L L A A V I 175
563 AACACGCCACCCAAGGCCGTAGCAACCACTCTGCCTATCAAATCCCCATTTCTATTCAGTTTGTCTGGGCATT
   N N A T Q G R S N H S A Y Q I P I S I O F V W A F 200
638 ATCCTTGGGTTTGGCATGATGCTTTTGGCGGAATCCCCCGTTTTCTCGTTAGGCAAGGTCGCGACGAAGATGCT
   I L G F G M M L L P E S P R F L V R Q G R D E D A 225
713 GCCCTGTCTTGTCCCGTCTTACGAGGCTCTCGCCTGATGACCCCGTTGTGAGAAATGAATTGGCCGAGATTTCGC
   A L S L S R L T R L S P D D P V V R N E L A E I R 250
788 ACCAATTTTGGAGGCTGAACCTGGCCCTCGGTGAAAGCTCGTACTTGGATTGCTTCAGACCTCGCACAAACAAATT
   T N F E A E L A L G E S S Y L D C F R P S H N K I 275
863 TTGTTGCGTACCCTGACTGGTATTTTCATTCAAGCTTGGCAACAACCTTACGGGCATCAACTTTATTTTCTACTAT
   L L R T L T G I F I Q A W O O L T G I N F I F Y Y 300
938 GGTACACAATACTTCCAGAAGCTGGCATCAATCAAGCATTTCCTCATTACCATTGCCACCGGTATCGTCAACGTA
   G T Q Y F Q N A G I N Q A F L I T I A T G I V N V 325
1013 TTCATGACATTACCGGGCTTGTATGGTGTGCAACGCTATGGTTCGCCGTTCCCTCCTCCTTATTGGTGCCTAGGC
   F M T G L P G L Y G V E R Y G R R S L L L I G A L G 350
1088 ATGACGATTTGCGAGTTTATTGTTGCTATCGTTGGTGTGACGATCCCGCCTCGAATCTAGCAGGTGACAGAGTG
   M T I C E F I V A I V G V T I P A S N L A G Q R V 375
1163 CTAATTGCATTTCGTTTGCATTTACATCGCTTTTCTTCGCATCTACCTGGGGTCTTATGCTTTGGGTTATCGTTGGT
   L I A F V C I Y I A F F A S T W G P M L W V I V G 400
1238 GAAATCTTTCTCTACAAGTCCGTGCCAAGGGCATTTTCGCTTTCTGTTGCCAGCAACTGGTTGTGGAACCTTCGGT
   E I F P L Q V R A K G I S L S V A S N W L W N F G 425
1313 ATTGCTTTTGTCTACACCTTACCTTGTAAATACCCAAGCGGGATCAGCCGGTCTCGGGTCAAAAGTGTCTTTCATC
   I A F A T P Y L V N T Q A G S A G L G S K V F F I 450
1388 TGGGGCACAACATGTGCTTGTCTTGTCTTTACGTACTTCTGCGTGCCCGAGACCAAGGGCTTGTCTCTCGAA
   W G T T C A C C L V F T Y F C V P E T K G L S L E 475
1463 CAGATTGACGAGATGTATCAGGAAGTAATGCCATTAAAATCCTACCAATACCGCCGACGCTCGCGATAGAAGGC
   Q I D E M Y Q E V M P L K S Y Q Y R R R L A I E G 500
1538 GGATTTAGGACAGAAAAGGCTGATATCATACAGAGGAAACGGCAGAAAAGGAAAGCGGGTAACAGTTTCTACCC
   G F R T E K A D I I H E E T A E K E S G 520
1613 TTGTATCATGTTTTTCTCCACGTAATTAGTTCTTCAGACTTGTGATACAAAATGTCATCGTAAGTTACTTGAAGC
1688 CGCCCCAAAAAAAAAAAAAAAAAAAA

```

Fig. 2. cDNA and deduced protein sequences of AmMst1. Numbers on left side represent position of the nucleotides in the cDNA; those on right side, position of the amino acids of the deduced protein sequence. Putative membrane-spanning α -helices, predicted according to Kyte and Doolittle (1982), are underlined, primer binding sites used for polymerase chain reaction amplification of the initial AmMst1 cDNA fragment are double-underlined.

In contrast, symbiotic interaction led to a fourfold enhancement of gene expression (Fig. 6A). This experiment was conducted three times with RNA of different mycorrhizal samples. It was taken into account (see Material and Methods for detail) that in *P. abies*/*A. muscaria* mycorrhizas fungal and plant RNAs are present in a ratio of 1:1 (U. Nehls, *unpublished*). Enhanced AmMst1 expression in mycorrhizas could be confirmed with aspen as the plant partner (Fig. 6B). Furthermore, cultivation of mycelia in suspension culture at high glucose and fructose concentrations (40 mM) also increased the amount of AmMst1 transcript to a level comparable to that found in mycorrhizas (Fig. 6B). Enhanced AmMst1 expression did not occur in fruiting bodies of *A. muscaria* (Fig. 6B), where high internal glucose concentrations are present (Wallenda 1996).

To determine the effect of monosaccharide concentration on AmMst1 expression in detail, increasing glucose and fructose concentrations were added to mycelia precultivated with 1

mM glucose (Fig. 7). Monosaccharide concentrations of up to 3 mM in the growth medium had only little effect on AmMst1 expression, whereas concentrations above 5 mM led to a fully enhanced AmMst1 gene expression.

Time course of AmMst1 expression.

A. muscaria mycelia were precultivated at a glucose concentration of 1 mM. The mycelia were then transferred to a medium containing 40 mM glucose, and cultivated for up to 5 weeks. Mycelial samples were taken at different times, total RNA was prepared, and Northern blots were carried out (Fig. 8). For the first 18 h of cultivation, no changes in AmMst1 expression were observed. Enhanced gene expression became visible after 1 day of fungal growth at high glucose concentrations. Thereafter, the degree of AmMst1 gene expression remained constant through day four. After 8 days of growth, the glucose concentration in the growth medium had decreased to 3.6 mM, and the AmMst1 transcript level returned

Fig. 3. Cladogram of AmMst1 and other fungal monosaccharide transporters. Relationship of the deduced protein sequence of AmMst1 to other fungal monosaccharide transporters was determined by multiple alignment with Clustal W (version 1.5). Identity and similarity of monosaccharide transporter proteins (underlined symbols) representing different homology groups to the deduced AmMST1 protein are given in Table 1.

to the initial level. Even complete consumption of the supplied glucose after 16 days as well as an additional 16 days of fungal culture without glucose did not result in a further change of AmMst1 expression.

Effect of different carbohydrates and glucose analogues on AmMst1 expression.

To investigate whether AmMst1 expression is regulated by glucose itself or by products of glucose metabolism, fungal mycelia were precultivated in 1 mM glucose for 1 week. The mycelia were transferred to a medium containing 40 mM of the glucose analogues 3-*O*-methyl-glucose and 2-deoxyglucose, or sorbitol, mannitol, gluconate, or arabinose. The mycelia were cultivated for 2 (Fig. 9) or 7 days (data not shown) under these conditions. Neither of the glucose analogues (3-*O*-methyl-glucose and 2-deoxyglucose) led to an enhancement of AmMst1 expression. Likewise, cultivation on mannitol, sorbitol, or gluconate as carbon sources revealed only a basal expression of AmMst1, whereas incubation with arabinose or ribose (data not shown) yielded transcript levels similar to those obtained with 40 mM glucose.

DISCUSSION

Properties of the *A. muscaria* monosaccharide transporter.

For the initial isolation of monosaccharide transporter genes of *A. muscaria* a PCR-based strategy was used. Primers were designed against two amino acid stretches highly conserved in most of the known fungal monosaccharide transporters. The use of these primers for reverse transcription (RT)-PCR amplification of mycorrhizal mRNA resulted in the identification of only one gene, AmMst1. An independent approach, with a different set of primers and cDNA from fungal mycelium grown at elevated glucose concentrations on agar medium, also resulted in the identification of the same AmMst1 gene (M. Guttenberger, *unpublished results*). Screening of the mycorrhizal cDNA library with AmMst1 cDNA as a probe did not indicate any additional genes related to AmMst1, even under low-stringency conditions of hybridization. These results indicate that no other related monosaccharide transport-

ers are expressed at a level comparable to that of AmMst1 in ectomycorrhizas. Furthermore, by Southern blot analysis, no hints were found for additional monosaccharide transporters homologous to AmMst1. Altogether, these results strongly suggest that AmMst1 represents the major, if not the only, monosaccharide transporter expressed in *A. muscaria* mycorrhizas as well as in hyphae grown at elevated external glucose concentrations. This assumption does not exclude the presence of further monosaccharide transporters expressed at a low level in mycorrhizas, or high-affinity monosaccharide transporters expressed mainly at low external monosaccharide concentrations and sharing limited homology with AmMst1.

In comparison, yeast has more than 20 monosaccharide transporter genes, and a number of these genes were identified with heterologous yeast probes (Reifenberger et al. 1995). On the other hand, *S. pombe*, for example, contains only one glucose uptake system. Monosaccharide uptake systems are also present in other fungi (for a review see Jennings 1995), but in most cases the actual number of monosaccharide transporters present in these systems is unknown.

Regulation of the AmMst1 gene expression.

A. muscaria hyphae grown at glucose concentrations lower than 5 mM expressed the AmMst1 gene at a basal level, while monosaccharide concentrations above 5 mM triggered a four-fold increase of the amount of the AmMst1 transcript. Thus,

Table 1. Homology of AmMST1 to known monosaccharide transporters^a

Gene	Organism	Identity	Similarity
RCO3	<i>Neurospora crassa</i>	45	63
RGT2	<i>Saccharomyces cerevisiae</i>	41	65
SNF3	<i>S. cerevisiae</i>	41	62
HXT4	<i>S. cerevisiae</i>	39	57
HXT5	<i>S. cerevisiae</i>	39	57
HXT1	<i>S. cerevisiae</i>	39	58
HXT8	<i>S. cerevisiae</i>	38	55
HXT4	<i>S. cerevisiae</i>	38	55
GAL2	<i>S. cerevisiae</i>	37	56
HXT1	<i>S. cerevisiae</i>	36	56
HXT1	<i>S. cerevisiae</i>	36	56
HXT2	<i>S. cerevisiae</i>	36	54
HXT9	<i>S. cerevisiae</i>	30	51
STL1	<i>S. cerevisiae</i>	28	46

^a The homology of AmMST1 to single monosaccharide transporters representing different homology groups (see Figure 3) was determined by the program Clustal W (Thompson et al. 1994). Identity and similarity (identical amino acids plus conserved amino acid exchanges) were calculated.

Fig. 4. Determination of the copy number of the AmMst1 gene. Genomic DNA was isolated from *Amanita muscaria* mycelia and samples of 15 µg of DNA each were digested with different restriction enzymes. DNA fragments were separated on a 0.6% agarose gel, transferred to a nylon membrane, and hybridized with the entire AmMst1 cDNA. Lane 1: *SalI*. Lane 2: *EcoRI*. Lane 3: *BamHI*. Lane 4: *KpnI*. Lane 5: *XbaI*. Lane 6: *HindII*. Lane 7: *XhoI*.

Fig. 5. Heterologous expression of AmMst1 cDNA in *Schizosaccharomyces pombe*. A monosaccharide transporter-deficient *S. pombe* mutant was transformed with constructs containing the AmMst1 cDNA in sense (♦) and antisense (Δ) orientation under control of the yeast ADH promoter and the control (X) (SAP-E vector only). AmMst1 cDNA in sense orientation was expressed under its own internal start ATG. Transformants were precultivated on medium containing gluconate for 1 week and were then transferred to a medium containing 100 mM glucose as carbon source. Growth rates (OD₅₇₈) of transformed yeast strains on glucose are shown.

Fig. 6. AmMst1 transcript levels under different developmental and growth conditions. AmMst1 expression level was compared in fungal mycelium grown at low (1 mM) or elevated (40 mM) monosaccharide concentrations, in mycorrhizas and fruiting bodies of *Amanita muscaria*. Total RNA was extracted, separated on an agarose gel, transferred to nylon membranes, and hybridized with either AmMst1 cDNA or *A. muscaria* 5.8 S rRNA. **A**, Lane 1: nonmycorrhized *Picea abies* roots. Lane 2: *P. abies*/*A. muscaria* mycorrhizas. Lane 3: *A. muscaria* mycelium grown with 1 mM glucose as carbon source on agar plates or (Lane 4) in liquid culture. **B**, Lane 5: gills of fruiting bodies. Lane 6: fruiting bodies without gills. Lane 7: *P. abies*/*A. muscaria* mycorrhizas. Lane 8: *Populus tremula* × *tremuloides*/*A. muscaria* mycorrhizas. Lane 9: *A. muscaria* mycelium grown with 40 mM glucose. Lane 10: *A. muscaria* mycelium grown with 40 mM fructose.

we assume that this change in AmMst1 expression is regulated by a threshold response mechanism depending on the extracellular concentration of monosaccharides.

The increase of AmMst1 expression is a slow process compared with the induction of the yeast high-affinity glucose transport system HXT2, which occurs within 90 min (Bisson and Fraenkel 1984). For AmMst1 a transition from basal to maximal expression rate occurred between 18 h and 1 day of fungal culture. This is possibly due to the fact that soil-grown mycelia are exposed to low concentrations of carbohydrates (Wainwright 1993). Only locally and for a very short time were higher monosaccharide concentrations found. Under such conditions, monosaccharides could be exploited by the basal rate of AmMst1 expression and/or by a monosaccharide uptake system preferentially expressed at low external monosaccharide concentrations. The extended lag phase for enhanced AmMst1 expression would avoid metabolic investment into a protein not needed on a long-term basis.

In contrast to soil-grown hyphae, hyphae at the symbiotic interface are exposed to a continuous supply of plant-derived carbohydrates making up to 30% of the photosynthetically fixed carbon (Söderström 1992). To maximize the carbohydrate flow into the fungus, monosaccharides must be quickly taken up and either metabolized or converted into compounds for intermediate or long-term storage (e.g., trehalose, mannitol, glycogen). In *A. muscaria* ectomycorrhizas, a fourfold increase of AmMst1 expression was observed, when compared with hyphae grown at low glucose concentrations. Increasing the number of monosaccharide transporters in the plasma membrane should significantly increase the overall capacity for monosaccharide uptake of hyphae in symbiosis. We therefore assume that both the extended lag phase for enhanced AmMst1 expression and its threshold response to elevated monosaccharide concentrations are adaptations of the ectomycorrhizal fungus to the permanent supply of carbohydrates found at the symbiotic interface.

Fig. 7. AmMst1 expression in mycelia grown at different monosaccharide concentrations. After precultivation of fungal mycelia at 1 mM glucose, mycelia were cultivated at different glucose or fructose concentrations (0.5 to 50 mM) for 2 days. Total RNA was extracted, separated on an agarose gel, transferred to nylon membranes, and hybridized with either AmMst1 cDNA or 5.8 S rRNA.

Fig. 8. Time course of AmMst1 expression. Glucose at a final concentration of 40 mM was added to mycelia precultivated at 1 mM glucose. Mycelial samples were taken after different incubation times and the actual glucose concentration in the medium was determined. Total RNA was extracted from the mycelia, separated on an agarose gel, and transferred to a nylon membrane. Membrane was hybridized with either AmMst1 cDNA or 5.8 S rRNA.

The enhancement of AmMst1 expression under high glucose conditions raises the question whether glucose transport, glucose phosphorylation, or other mechanisms are involved in glucose sensing. Therefore, we cultured *A. muscaria* mycelia in the presence of 40 mM 3-*O*-methyl-glucose or 2-deoxyglucose. Both glucose analogues are taken up by fungal hyphae (Chen and Hampp 1993; A. Wiess, *unpublished results*). While 3-*O*-methyl-glucose is only imported into hyphae, 2-deoxyglucose is both imported and subsequently phosphorylated by hexokinase but is not further metabolized (Allen et al. 1989; Jang and Sheen 1994). The presence of either glucose analogue failed to enhance AmMst1 expression, revealing that neither the transport of glucose into the cell nor its phosphorylation by a hexokinase is sufficient to trigger the described response. We assume that glucose-6-phosphate metabolism downstream of the hexokinase, e.g., carbohydrate storage pathways (trehalose or glycogen synthesis), might act as sugar sensor. This hypothesis is supported by findings of Thevelein and Hohmann (1995), who supposed trehalose-6-phosphate synthase together with a monosaccharide transporter as glucose sensor in yeast.

The *in vivo* concentration of monosaccharides at the fungus/plant interface is presumably not the only element controlling AmMst1 expression in mycorrhizas. Only hyphae of the Hartig net and the inner part of the fungal mantle, altogether approximately 30 to 50% of the fungal biomass in mycorrhizas (estimated according to mycorrhizal sections), are supposed to sense plant-derived monosaccharides. Taking into account that in the outer parts of the fungal mantle AmMst1 may be expressed at the basal level, the obtained fourfold overall enhancement of gene expression in mycorrhizas might represent an eight- to 10-fold enhancement of AmMst1 transcript level in hyphae that are in contact with the root cortex. Therefore, a symbiosis-regulated control mechanism could be involved in addition to sugar regu-

lation in mycorrhizas, resulting in further enhancement of AmMst1 expression.

MATERIALS AND METHODS

Biological material.

Amanita muscaria (L.) ex Fr. Hooker strain CS83 was isolated from fruiting bodies (Schaeffer 1995). Mycelia were grown in liquid culture for 2 to 32 days according to Chen and Hampp (1993) with various carbohydrates as carbon source. The mycelium was collected by filtration with a Büchner funnel under suction, washed twice with 50 ml of H₂O, frozen in liquid nitrogen, and stored at -80°C.

Seeds of *Picea abies* (L.) H. Karst were obtained from the Staatsklänge (Nagold, Germany). Nonmycorrhizal plants and mycorrhizas were obtained according to Guttenberger and Hampp (1992). Aspen (*Populus tremula* × *Populus tremuloides*)/*A. muscaria* mycorrhizas were obtained according to Hampp et al. (1996). Mycorrhizal and nonmycorrhizal lateral roots were harvested, frozen in liquid nitrogen, and stored at -80°C.

Isolation of RNA.

Frozen mycorrhizas, nonmycorrhizal lateral roots, and fungal mycelia were ground to a fine powder in a mortar with pestle under liquid nitrogen. Five volumes of a solution containing 4 M guanidine isothiocyanate, 96 mM β-mercaptoethanol, and 25 mM sodium acetate, pH 6.0, were added and the suspension transferred to a centrifuge tube. Insoluble material was removed by centrifugation (20 min, 20,000 × *g*, 20°C). The resulting supernatant was transferred to ultracentrifuge tubes and mixed with 1 volume of 5.7 M CsCl, 25 mM sodium acetate, pH 6.0. The RNA was sedimented by centrifugation (20 h, 200,000 × *g*, 20°C) and the supernatant was removed carefully. The pellet was resuspended in 200 µl of diethyl pyrocarbonate (DEPC)-treated H₂O, transferred to an Eppendorf tube, precipitated by the addition of 20 µl of 3 M sodium acetate and 500 µl of ethanol, and collected by centrifugation (40 min, 14,000 × *g*, 20°C). The RNA was resuspended in 50 to 100 µl of DEPC-treated H₂O. Aliquots were frozen in liquid nitrogen and stored at -80°C.

Northern blot analysis.

Northern blots were carried out according to Nehls and Martin (1995) with 5 to 15 µg of total RNA. In *P. abies*/*A. muscaria* mycorrhizas total RNA is a 1:1 mixture of both partners (U. Nehls, *unpublished*). Therefore, 15 µg of total RNA in mycorrhizal lanes was compared with 7.5 µg of RNA from the nonmycorrhizal fungal controls. The nonmycorrhizal fungal RNA was mixed with 7.5 µg of total RNA of *P. abies* nonmycorrhizal roots (that does not hybridize with the AmMst1 probe), to ensure that identical amounts of RNA were loaded into the different lanes. For the quantification of the signal intensities, a flatbed scanner (Scanmaker IIHR, Microtek, Dusseldorf, Germany) and the program package NIH Image (version 1.6; NIH Image is available on-line from the National Institutes of Health) were used.

PCR amplification and cloning of cDNA fragments.

Ten micrograms of total RNA was used for first-strand cDNA synthesis, performed in a volume of 20 µl with 200 U

Fig. 9. Effect of different sugars and glucose analogues on AmMst1 expression. Fungal mycelia were precultivated in liquid culture containing 1 mM glucose as carbon source for 4 days. Sugars were added to a final concentration of 40 mM and the mycelium was grown for an additional 2 days. Total RNA was extracted, separated on an agarose gel, transferred to a nylon membrane, and hybridized with the AmMst1 cDNA or *Amanita muscaria* 5.8S rRNA. Lane 1: 2-deoxyglucose. Lane 2: 3-*O*-methylglucose. Lane 3: gluconate. Lane 4: glucose. Lane 5: mannitol. Lane 6: sorbitol. Lane 7: arabinose.

of Superscript II (GibcoBRL, Eggenstein, Germany) reverse transcriptase according to the manufacturer's instructions. The cDNA was ethanol precipitated and resuspended in 10 µl of H₂O. Two microliters of the cDNA solution was used for an initial PCR (30 cycles: 45 s, 32°C; 1 min, 72°C; 1 min, 92°C; GeneAmp 2400 thermocycler, Perkin Elmer, Foster City, CA) in a total volume of 100 µl containing 4 U of *Taq* DNA polymerase (Eurogentec, Seraing, Belgium). 5' primer: (C/T)TICA(A/G)CA(A/G)(C/T)TIACIGG; 3' primer: A(G/A)I(C/G)(A/T)IA(A/T)IA(A/G)ICC(C/T)TTIGT(C/T)TC (I = inosine). Five microliters of the initial PCR product was used for a second PCR under the same conditions. The cDNA fragments of the second PCR were separated on an agarose gel, stained with ethidium bromide, and excised with a scalpel. The cDNA was isolated from the gel with a gel extraction kit (Qiagen, Hilden, Germany) and cloned into pGEM-T-vector (Promega, Mannheim, Germany).

Construction of an *S. pombe* expression vector.

The entire cDNA insert of AmMst1, initially cloned into pBluescript (Stratagene, Heidelberg, Germany), was excised by *Eco*RI and *Xho*I digestion, separated on an agarose gel, and isolated from the gel with a gel extraction kit (Qiagen). SapE (Truernit et al. 1996) was digested with *Eco*RI and both the entire cDNA and the linearized SapE vector were blunted with T4 DNA polymerase (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. The SapE vector was further dephosphorylated with shrimp alkaline phosphatase (Boehringer). Ligation of the AmMst1 cDNA in the dephosphorylated SapE vector was performed with T4 DNA ligase (Boehringer) for 16 h at room temperature. The construct was transformed into *Escherichia coli* and clones containing the AmMst1 insert were selected by the size of the isolated plasmid. The orientation of the AmMst1 within the vector was determined with a single, asymmetric, *Bam*HI restriction site within the cDNA.

Miscellaneous.

Glucose and fructose concentrations were assayed according to Einig and Hampp (1990). Genomic DNA was isolated according to Nehls et al. (1992) from 5 g (fresh weight) of mycelium. Subclones for sequencing of the cDNA were generated with the nested deletion kit (Pharmacia, Freiburg, Germany). Sequencing (Sanger et al. 1977) was performed with the T7 DNA-sequencing kit (Pharmacia), M13 primer (universal and reverse; Eurogentec), or SK primer (Stratagene). For analysis of DNA and protein sequences, the program package GeneJockey II (Biosoft, Cambridge, UK) was used on a Macintosh computer. The sequence data were compared to gene libraries (EMBL and GenBank) with Blastx (Altschul et al. 1990) and further analyzed with Clustal W 1.5 (Thompson et al. 1994).

ACKNOWLEDGMENTS

We thank Magret Ecke and Elke Klenk for excellent technical assistance, and Werner Einig and Ellen Hoffmann for critical reading of the manuscript. We are also indebted to W. Tanner, University of Regensburg, for helpful discussions and for the supply of the monosaccharide transporter-deficient *S. pombe* mutant. This work was supported by the Deutsche Forschungsgemeinschaft (Graduiertenkolleg "Organismische Interaktionen in Waldökosystemen").

LITERATURE CITED

- Allen, K. F., McNally, M. T., Lowendorf, H. S., Slayman, C. W., and Freff, S. J. 1989. Deoxyglucose-resistant mutants of *Neurospora crassa*: Isolation, mapping and biochemical characterization. *J. Bacteriol.* 171:53-58.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Bisson, L. F., and Fraenkel, D. G. 1984. Expression of kinase-dependent glucose uptake in *Saccharomyces cerevisiae*. *J. Bacteriol.* 159:1013-1017.
- Chen, X.-Y., and Hampp, R. 1993. Sugar uptake by protoplasts of the ectomycorrhizal fungus *Amanita muscaria*. *New Phytol.* 125:601-608.
- Einig, W., and Hampp, R. 1990. Carbon partitioning in Norway spruce: Amounts of fructose 2,6-bisphosphate and of intermediates of starch/sucrose synthesis in relation to needle age and degree of needle loss. *Trees* 4:68-74.
- Guttenberger, M., and Hampp, R. 1992. Ectomycorrhizins - symbiosis-specific or artifactual polypeptides from ectomycorrhizas? *Planta* 188:129-136.
- Hampp, R., Ecke, M., Schaeffer, C., Wallenda, T., Wingler, A., Kottke, I., and Sundberg, B. 1996. Axenic mycorrhization of wild type and transgenic hybrid aspen expressing T-DNA indoleacetic acid-biosynthetic genes. *Trees* 11:59-64.
- Hampp, R., and Schaeffer, C. 1995. Mycorrhiza - carbohydrate and energy metabolism. Pages 267-296 in: *Mycorrhiza*. A. Varma and B. Hock, eds. Springer-Verlag, Berlin.
- Harley, J. L., and Smith, S. E. 1983. *Mycorrhizal Symbiosis*. Academic Press, London.
- Jang, J. C., and Sheen, J. 1994. Sugar sensing in higher plants. *Plant Cell* 6:1665-1679.
- Jennings, D. J. 1995. *The Physiology of Fungal Nutrition*. Cambridge University Press, Cambridge.
- Kyte, J., and Doolittle, R. F. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105-132.
- Madi, L., McBridge, S. A., Bailey, L. A., and Ebbola, D. J. 1997. Rco-3, a gene involved in glucose transport and conidiation in *Neurospora crassa*. *Genetics* 146:499-508.
- Marger, M. D., and Saier, M. H. 1993. A major superfamily of transmembrane facilitators that catalyze uniport, symport and antiport. *Trends Biol. Sci.* 18:13-20.
- Melin, E., and Nilsson, H. 1957. Transport of ¹⁴C-labelled photosynthate to the fungal associate of pine mycorrhiza. *Sven. Bot. Tidskr.* 51:166-186.
- Milbradt, B., and Höfer, M. 1994. Glucose-transport-deficient mutants of *Schizosaccharomyces pombe*: Phenotype, genetics and use for genetic complementation. *Microbiology* 140:2617-2623.
- Nehls, U., and Martin, F. 1995. Gene expression in plant roots during ectomycorrhiza development. Pages 125-137 in: *Biotechnology of Ectomycorrhizae: Molecular Approaches*. V. Stocchi, ed. Plenum Publishing, New York.
- Nehls, U., Friedrich, T., Schmiede, A., Ohnishi, T., and Weiss, H. 1992. Characterization of assembly intermediates of NADH: Ubiquinone oxidoreductase (complex I) accumulated in *Neurospora* mitochondria by gene disruption. *J. Mol. Biol.* 227:1032-1042.
- Reifenberger, E., Freidel, K., and Ciriacy, M. 1995. Identification of novel HXT genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. *Mol. Microbiol.* 16:157-167.
- Salzer, P., and Hager, A. 1991. Sucrose utilization of the ectomycorrhizal fungi *Amanita muscaria* and *Hebeloma crustuliniforme* depends on cell wall-bound invertase activity of their host *Picea abies*. *Bot. Acta* 104:439-445.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Schaeffer, C., Wallenda, T., Guttenberger, M., and Hampp, R. 1995. Acid invertase in mycorrhizal and non-mycorrhizal roots of Norway spruce (*Picea abies* (L.) Karst) seedlings. *New Phytol.* 129:417-424.
- Smith, S. E., and Read, D. J. 1997. *Mycorrhizal Symbiosis*. 2nd ed. Academic Press, London.
- Söderström, B. 1992. The ecological potential of the ectomycorrhizal mycelium. Page 77-83 in: *Mycorrhizas in Ecosystems*. D. G. Read, D. H. Lewis, A. H. Fitter, and I. J. Alexander, eds. CAB Int., Wallingford, U.K.

- Thevelein, J. M., and Hohmann S. 1995. Trehalose synthase: Guard to the gate of glycolysis in yeast? *Trends Biolog. Sci.* 20:3-10.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680.
- Truernit, E., Schmid, J., Eppe, P., Illig, J., and Sauer, N. 1996. The sink-specific and stress-regulated *Arabidopsis* STP4 gene: Enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge. *Plant Cell* 8:2169-2182.
- Wainwright, M. 1993. Oligotrophic growth of fungi - stress or natural state. Pages 127-144 in: *Stress Tolerance of Fungi*. D. J. Jennings, ed. Marcel Dekker, New York.
- Wallenda, T. 1996. Untersuchungen zur Physiologie der Pilzpartner von Ektomykorrhizen der Fichte. Ph.D. thesis. Universität Tübingen, Tübingen, Germany.