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A eukaryotic melibiose transporter

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Journal research area

Plants interacting with other organisms
Functional characterization of a eukaryotic melibiose transporter

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ABSTRACT

Pathogenic fungi drastically affect plant health and cause significant losses in crop yield and quality. In spite of their impact, little is known about the carbon sources used by these fungi in planta and about the fungal transporters importing sugars from the plant-fungus interface. Here, we report on the identification and characterization of MELIBIOSE TRANSPORTER1 (MBT1) from the hemibiotrophic fungus Colletotrichum graminicola (teleomorph Glomerella graminicola), the causal agent of leaf anthracnose and stalk rot disease in maize (Zea mays L.). Functional characterization of the MBT1 protein in baker’s yeast (Saccharomyces cerevisiae) expressing the MBT1 cDNA revealed that α-D-galactopyranosyl compounds such as melibiose, galactinol and raffinose are substrates of MBT1, with melibiose most likely being the preferred substrate. α-D-Glucopyranosyl disaccharides like trehalose, isomaltose or maltose are also accepted by MBT1, although with lower affinities. The MBT1 gene shows low and comparable expression levels in axenically grown C. graminicola and upon infection of maize leaves both during the initial biotrophic development of the fungus and during the subsequent necrotrophic phase. Despite these low levels of MBT1 expression, the MBT1 protein allows efficient growth of C. graminicola on melibiose as sole carbon source in axenic cultures. Although Δmbt1 mutants are unable to grow on melibiose, they do not show virulence defects on maize.
INTRODUCTION

In a search for neutral, non-permeating osmolytes suitable for studies involving the response of suspension-cultured tobacco cells (*Nicotiana tabacum*) to water deficit, Dracup et al. (1986) identified the disaccharide melibiose (\(\alpha\)-D-Galp-1,6-D-Glcp) as a useful compound. Other molecules tested were either actively imported into the plant cells, such as sorbitol and mannitol, or hydrolyzed in the apoplast, such as raffinose (\(\alpha\)-D-Galp-1,6-\(\alpha\)-D-Glcp-1,2-\(\beta\)-D-Fruf). Raffinose hydrolysis was mediated by cell wall-bound invertases (\(\alpha\)-D-Glcp-1,2-\(\beta\)-D-Fruf; Roitsch and González, 2004; Vargas et al., 2009), but of the resulting products, only fructose was taken up by the plant cells whereas melibiose accumulated in the medium. In fact, although suspension cultures of other plants including sugar cane (*Saccharum* spec.; Nickell and Maretzki, 1970) or carrot (*Daucus carota*; Verma and Dougall, 1977) were shown to use melibiose as sole carbon source, probably after extracellular hydrolysis by \(\alpha\)-galactosidases, their growth rates on this disaccharide were extremely low suggesting that extracellular melibiose is not a substrate for plant transporters. Melibiose was, however, identified as an intracellular metabolite in soil-grown Arabidopsis plants (Fiehn et al., 2000).

Plasma membrane-localized disaccharide transporters of plants catalyze the uptake of sucrose or maltose (\(\alpha\)-D-Glcp-1,4-\(\alpha\)-D-Glcp) (Sauer, 2007), but proteins transporting raffinose or melibiose have so far not been identified. On the other hand, numerous cell wall-localized \(\alpha\)-galactosidases that might hydrolyze these molecules in the apoplast were identified from different plant species (Feurtado et al., 2001; Pennycooke et al., 2003; Chrost et al., 2007). Together with the growth analyses mentioned above, this might indicate that, unlike sucrose, raffinose and melibiose
typically are intracellular carbohydrates and not intentionally released into the plant apoplast for the supply of adjacent cells or tissues.

This changes, however, during specific symbiotic interactions. When Bringhurst et al. (2001) studied the activity of a galactoside-sensing α-galactosidase-GFP (green fluorescent protein) construct in the transgenic soil bacterium Sinorhizobium meliloti in the presence of root washes from different legume species, they observed strong GFP fluorescence in S. meliloti. Qualitative analyses of these root washes identified legume-derived secreted raffinose and stachyose (\(\alpha-D-Galp-1,6-\alpha-D-Galp-1,6-\alpha-D-Glc\)p-1,2-\(\beta-D-Fru\)f) as the inducing compounds suggesting that these oligosaccharides are secreted to feed rhizosphere symbionts. In fact, a raffinose uptake system (Mrt = Metarhizium raffinose transporter) was identified in the mutualistic soil fungus Metarhizium robertsii (Fang and Leger, 2010) that allows this fungus to grow on different di- and oligosaccharides including sucrose, lactose (\(\beta-D-Galp-1,4-D-Glc\)p), raffinose, stachyose or melizitose (\(\alpha-D-Glc\)p-1,3-\(\beta-D-Fru\)f-2,1-\(\alpha-D-Glc\)p).

Di- and oligosaccharides are attractive carbon sources also for plant pathogenic fungi. While biotrophic fungi only penetrate the cell wall of their host but not the plasma membrane to grow extracellularly, fungi exhibiting a necrotrophic lifestyle invade the plant cell and kill the host by secretion of toxins or by generating reactive oxygen species (ROSs; Mendgen and Hahn, 2002; Horbach et al., 2011). Thus, depending on their lifestyle, pathogens have access to different carbon sources. The biotrophic fungus Ustilago maydis, the causal agent of corn smut, has a plasma membrane-localized sucrose transporter, Srt1, that enables this fungus to feed on apoplastic sucrose (Wahl et al., 2010). The srt1 gene is expressed exclusively after infection of plant tissue, and, surprisingly, the presence of this gene is essential for suppression of host defense by U. maydis and for virulence. It is important to note
that biotrophic fungi have fewer genes encoding cell wall-degrading enzymes (Kämper et al., 2006) and cause only minimal cell wall damage during the infection process (Mendgen and Deising, 1993).

We searched for genes encoding putative di- and/or oligosaccharide transporters in the genome of the hemibiotrophic maize (Zea mays) pathogen Colletotrichum graminicola (Cesati) Wilson [teleomorph Glomerella graminicola (Politis)], the causal agent of the worldwide occurring stem rot and leaf anthracnose (Münch et al., 2008; Horbach et al., 2011). After a short biotrophic growth phase of 48 to 72 h in the apoplast, C. graminicola initiates its necrotrophic development by secreting cell wall-degrading enzymes, breaching the plant plasma membrane of its host, and gaining access to cell wall-derived and intracellular carbon sources that are not available to biotrophic fungi.

Here we report on the identification and functional characterization of MELIBIOSE TRANSPORTER 1 (MBT1), a plasma membrane-localized protein from C. graminicola. The MBT1 gene is expressed after infection of maize leaves and in sterile cultures of C. graminicola on glucose medium and/or on a medium containing cell wall fragments and extracts from uninfected maize leaves (Krijger et al., 2008), which is thought to mimic the plant environment. Expression of MBT1 in baker’s yeast (Saccharomyces cerevisiae) characterized MBT1 as a high-affinity transporter that transports preferably α-galactosides with melibiose being its best substrate. To our knowledge, melibiose transport systems were so far described exclusively in bacteria. While Δmbt1 mutants of C. graminicola are unable to grow on melibiose as sole carbon source, the virulence of these strains was not affected.
RESULTS

Identification of MBT1 and Sequence Analyses

BLAST searches with AGT (α-glucoside transporter) sequences from *S. cerevisiae* and other fungi in the NCBI trace archives and in the *C. graminicola* genome database [http://www.ncbi.nlm.nih.gov/blast/Blast.cgi (Colletotrichum -WGS) and http://www.broadinstitute.org/annotation/genome/colletotrichum_group/MultiHome.html] identified 5 genes for proteins with significant homology to known or putative AGT transporters. The phylogenetic tree presented in Figure 1 demonstrates that these 5 sequences, which were initially named CgAGT1 to CgAGT5, are more closely related to fungal monosaccharide transporters than to plant disaccharide transporters. Moreover, the CgAGT cluster is closely linked to the well-characterized Mal11p protein from *S. cerevisiae*, a high-affinity maltose transporter (Chang and Michels, 1991) and it includes the functionally uncharacterized Mrt transporter from *M. robertsii* (Fang and Leger, 2010). Here we focus on the CgAGT1 protein, for which the phylogenetic tree in Figure 1 revealed closely related proteins in other plant pathogenic fungi. The protein was named MBT1 after its functional characterization.

MBT1 is a 611-amino acid (aa) protein with 12 predicted transmembrane helices and a deduced molecular mass of 67.62 kDa. The comparison of MBT1 genomic (GenBank accession #: FN433107) and cDNA sequences (accession #: FN433108) revealed that the gene is interrupted by a single, 188-nucleotide (nt) intron after the 391st nt of the coding sequence. BLAST searches with the MBT1 protein sequence identified MBT1-related proteins in other plant pathogenic fungi (Fig. 1), such as *Verticillium albo-atrum* [maltose permeases MAL31 (accession #: EEY19969) and MAL61 (accession #: EEY23935)], *Grosmannia clavigera* (uncharacterized protein: accession #: EFX01607), *Gibberella zeae* (uncharacterized
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protein: accession #: XP_380246) or Magnaporthe grisea (uncharacterized protein: accession #: EDJ94788). These proteins share between 54.6% (EDJ94788) and 65.6% (MAL31) identical amino acids with MBT1. Except for the V. albo-atrum MAL61 (60.3% identity) and the uncharacterized G. clavigera transporter (59.2% identity), however, the putatively cytoplasmic N-termini of all other proteins are significantly shorter (60 to 70 aa) than the N-terminus of the C. graminicola MBT1 protein.

The position of the single 188-nt intron in the MBT1 gene is conserved in the V. albo-atrum MAL61 gene (single 58-nt intron) and the G. clavigera gene (single 55-nt intron) underlining the similarity between these 3 genes. Analyses of the genomic sequences of these genes revealed another interesting feature. In the 5'-untranslated region (UTR) of the MBT1 gene we detected an upstream open reading frame (uORF) that starts at -19 and encodes a 3-aa peptide (Met-Val-Thr). Interestingly, analyses of the 5’-UTRs of the MBT1-related genes identified 4 potential uORFs in the closely related G. clavigera gene (starting at -8, -28, -50, -59). Such uORFs are known to strongly affect the expression of the following gene (Vilela and McCarthy, 2003).

**MBT1 is a Functionally Active Transporter and is Targeted to the Plasma Membrane in Baker’s Yeast**

For functional analyses, the MBT1 ORF was expressed with or without the 24 nt of its 5’-flanking sequence, i.e. with or without its uORF, in yeast strain SEY2102, which lacks the cell wall-bound invertase (Emr et al., 1983). The resulting strains were named ULY24 (MBT1 in sense orientation + uORF) and ULY38 (MBT1 in sense orientation - uORF) or UL24as and ULY38as (same inserts in antisense orientation).
We first studied the transport capacity of MBT1 for sucrose and maltose. Figure 2A demonstrates that the uptake rates for \(^{14}\text{C}\)-labeled sucrose (1 mM) were almost the same in ULY24 cells (MBT1 + uORF) and in the corresponding antisense strain (negative control). For comparison, cells expressing the cDNA of the \textit{U. maydis} sucrose transporter SRT1 (positive control; Wahl et al., 2010) showed high transport rates for sucrose under these conditions indicating (i) that either sucrose is a poor or no substrate for MBT1, (ii) that MBT1 is not or only weakly expressed in \textit{S. cerevisiae}, or (iii) that MBT1 is not targeted to the \textit{S. cerevisiae} plasma membrane. The two last points could be excluded by the transport analyses with \(^{14}\text{C}\)-labeled maltose (1 mM). \(^{14}\text{C}\)-Maltose was imported at significantly higher rates into MBT1 expressing cells than into antisense controls (Fig. 2B,C), and the removal of the uORF led to an additional strong increase in the maltose transport rates of MBT1 expressing cells (Fig. 2C). This demonstrated that the MBT1 ORF is expressed in \textit{S. cerevisiae}, that the encoded protein is targeted to the plasma membrane, and that maltose is a substrate of MBT1. Moreover, this result suggests that the uORF has a negative impact on the level of MBT1 expression.

**MBT1 is an \(\alpha\)-Galactosides Transporter with Preference for Melibiose**

In order to identify other substrates of MBT1, we tested the effects of candidate substrates on the uptake of \(^{14}\text{C}\)-labeled maltose. Figure 3A shows that in the presence of equimolar concentrations of unlabeled putative competitors strong inhibition was observed only with the \(\alpha\)-galactosides raffinose, galactinol and melibiose, with melibiose clearly causing the most pronounced inhibition. Of all other tested substrates, only the \(\alpha\)-glucoside trehalose (\(\alpha\)-D-Glc\(_p\)-1,1-\(\alpha\)-D-Glc\(_p\)) had a significant inhibitory effect. When the same analyses were performed with a 10-fold molar excess of the putative inhibitors (Fig. 3B), the inhibitory effects of these 4
compounds became even more obvious, and less pronounced inhibition was also observed with the α-glucosides isomaltose (α-D-Glc\(_p\)-1,6-D-Glc\(_p\)) and isomaltulose (α-D-Glc\(_p\)-1,6-D-Fru\(_f\)).

As the competition analyses shown in Figure 3A,B had been performed in ULY24 cells (\(MBT1 + uORF\)), selected competition analyses were repeated in ULY38 cells (\(MBT1 - uORF\); Fig. 3C). The results obtained from these analyses were essentially the same as those obtained with ULY24 cells (Fig. 3A,B) demonstrating that the absence or presence of the uORF does not affect the substrate specificity of MBT1.

The strong inhibition of MBT1-mediated maltose uptake into \(S.\ cer\ viesiae\) cells by the α-galactosides raffinose, galactinol and especially melibiose might be caused by the transport of these compounds but also simply by a competitive binding to the transporter without a subsequent transport step. To discriminate between these two options, we aimed to perform additional transport assays with at least one of these α-galactosides. As radiolabeled galactinol and melibiose are not commercially available, we performed additional uptake analyses with \(^3\)H-labeled raffinose (Fig. 4). As for maltose (Fig. 2B,C) these tests revealed uptake of \(^3\)H-raffinose only into \(MBT1\)-expressing \(S.\ cer\ viesiae\) cells but not into \(MBT1\)-antisense control strains (Fig. 4A,B). Moreover, significantly more raffinose (almost 20-fold) was taken up by cells expressing \(MBT1\) without its uORF (Fig. 4B) than by cells expressing \(MBT1\) with its uORF (Fig. 4A). In summary, this demonstrated that MBT1 does, in fact, catalyze the transmembrane transport of α-galactosides, and it confirmed the higher transport rates of ULY38 cells (\(MBT1 - uORF\)) that were already observed with maltose (Fig. 2B,C).

To confirm or disprove the predicted transport capacities for other α-galactosides such as melibiose or galactinol and for trehalose (Fig. 3), we incubated...
ULY38 cells (\textit{MBT1} - uORF) and ULY38as cells (antisense control) with different unlabeled substrate for 1 h at 29°C and determined the intracellular concentrations of these different substrates. Figure 5 shows that all tested α-galactosides, i.e. melibiose, galactinol and raffinose (positive control), are transported by MBT1 into ULY38 cells (\textit{MBT1} - uORF). The intracellular levels of these compounds were increased only in ULY38 cells but not in ULY38as controls. Moreover, trehalose, a compound found in \textit{S. cerevisiae} cells under all physiological conditions, is also transported by MBT1, as significantly higher trehalose concentrations were detected in ULY38 cells than in ULY38as controls (Fig. 5). Most importantly, all substrates tested were accumulated to intracellular concentrations clearly above the concentration equilibrium (dotted line in Fig. 5; e.g. more than 12-fold accumulation of melibiose) suggesting that MBT1 catalyzes the active uptake of its different substrates.

We next studied the pH-dependence of MBT1-driven raffinose transport (Fig. 6A), its sensitivity to cyanide-\textit{m}-chlorophenylhydrazone (CCCP; Fig. 6B,C), an uncoupler of transmembrane H\textsuperscript{+}-gradients, and its sensitivity to the SH-group inhibitor \textit{p}-chloromercuribenzenesulfonic acid (PCMBS; Fig. 6B), a compound widely used to inhibit the activity of disaccharide transporters in plants (M'Batchi and Delrot, 1984). Figure 6A shows that MBT1 has an optimum at pH 6.0 with a steep decline towards more basic pH values. The inhibitor analyses revealed no effect of PCMBS (50 µM) on MBT1-mediated raffinose transport at pH 6.0, and only a less than 40% reduction of the transport rates by CCCP (50 µM). This inhibition by CCCP was independent of the presence (Fig. 6B) or absence (Fig. 6C) of the uORF. The CCCP sensitivity was also studied at pH 5.0, where the transport rates were reduced by 60% (not shown).
**MBT1 is a High-Affinity Transporter**

The competition analyses shown in Figure 3 and the transport analyses with raffinose shown in Figures 4 and 5 characterized MBT1 as a transporter that prefers α-galactosides (melibiose > galactinol > raffinose) but also accepts several α-glucosides (trehalose >>> isomaltose, isomaltulose >> maltose etc.). To support this result with more rigorous quantitative data, we determined the substrate affinities of MBT1 for raffinose and maltose (Fig. 7). As suggested by the competition analyses, the affinity of MBT1 for raffinose (K_m-value: 0.31 ± 0.1 mM; Fig. 7B) was significantly higher (more 25-fold) than that for maltose (K_m-value: 8.7 ± 3.7 mM; Fig. 7A). Again, the absence or presence of the uORF had no effect on the substrate affinity of MBT1 [K_m-raffinose_{(+ uORF)}: 0.31 ± 0.1 mM; K_m-raffinose_{(- uORF)}: 0.42 ± 0.09 mM; Fig. 7B,C] but strongly affected the v_{max} values. Taken together, these data explain the reduced transport of ¹⁴C-maltose in the presence of equimolar or 10-fold higher concentrations of raffinose (Fig. 3B,C).

**MBT1 mRNA Abundance in C. graminicola is Low Under All Growth Conditions**

To study the expression of MBT1 in *C. graminicola* and to identify parameters potentially affecting this expression, we determined the mRNA abundance in *C. graminicola* cells grown in different liquid culture media (Fig. 8A), in *C. graminicola* cells grown on solid media supplemented with different α-galactosides (Fig. 8B), and in *C. graminicola* infection hyphae grown within infected maize leaves at different times after infection (days post infection, dpi; Fig. 8C). Different MBT1 mRNA levels were observed under all growth conditions (Fig. 8A to 8C). However, the abundance of MBT1 mRNA was very low under all conditions analyzed. In semiquantitative RT-PCR analyses, faint bands were observed only after a minimum of 40 PCR cycles (not shown). In fact, even the highest MBT1 levels were only about 1% of the level...
detected for the mRNA of the previously characterized *HXT3* gene, which encodes a plasma membrane hexose transporter (Fig. 8A to 8C; Lingner et al., 2011).

The data presented in Figures 8A and 8B show only minor responses of the *MBT1* mRNA levels to the addition of glucose, extracts or α-galactosides. Interestingly, however, the addition of each organic carbon source tested here results in a reduction of *MBT1* mRNA levels. In contrast, the *MBT1* mRNA levels increase during pathogenic growth in infected maize leaves (Fig. 8C).

**The Physiological Role of MBT1 in *C. graminicola***

To study the physiological role of MBT1 in *C. graminicola*, we generated two independent deletion strains (Δ*mbt1* #1 and Δ*mbt1* #2) that had the *MBT1* gene replaced by a hygromycin resistance cassette via homologous recombination. Deletion of the *MBT1* gene was confirmed by genomic Southern blot analyses (Fig. 9A). We also included a strain (Ect) that had the hygromycin resistance cassette ectopically inserted and its *MBT1* gene still intact (Fig. 9A). Comparative growth analyses of wild type (WT) *C. graminicola*, of the Δ*mbt1* #1 and Δ*mbt1* #2 mutants, and of the Ect control strain on solid medium supplemented with different carbon sources revealed no growth differences on the mono-, di-, and trisaccharides glucose, sucrose, maltose and raffinose or on the polysaccharides starch, pectin and cellulose (Fig. 9B). Interestingly, however, and in line with the characterized function of MBT1, the Δ*mbt1* #1 and Δ*mbt1* #2 mutants were unable to grow on melibiose as sole carbon source. In contrast, WT *C. graminicola* and the Ect control grew equally well on melibiose-supplemented medium (Fig. 9B).

When we used the *C. graminicola* WT strain, the Δ*mbt1* #1 and Δ*mbt1* #2 mutants, and the Ect control for virulence assays, we did not observe any macroscopic differences on detached maize at 4 dpi (Fig. 9C). We also could not
detect differences in the pathogenic development of the four different strains, when we studied their rates of germination, formation of melanized or non-melanized appressoria, and their penetration rates (Fig. 9C).

DISCUSSION

*C. graminicola* can grow saprophytically on a wide range of different compounds, but in the presence of its appropriate plant host, i.e. maize, it develops a hemibiotrophic lifestyle and uses apoplastic carbon sources during its initial biotrophic development and potentially the entire cellular content, including cell wall degradation products, during the final necrotrophic growth. In this study, we investigated a novel transport protein from *C. graminicola* and characterized it as a transporter for the disaccharide melibiose. So far, melibiose transporters were identified exclusively in prokaryotes, e.g. in *Salmonella typhimurium* (Tsuchiya et al., 1982), *Escherichia coli* (Yazyu et al., 1984; Burstein and Kepes, 1985), *Klebsiella pneumoniae* (Hama and Wilson, 1992) or *Enterobacter cloacae* (Okazaki et al., 1995). These proteins are promiscuous with respect to their co-transported ion (Na⁺, Li⁺, or H⁺) and belong to the so-called galactoside-pentoses-hexuronides (GPH) family of membrane proteins (Reizer et al., 1994; Poolman et al., 1996; Ganea and Fendler, 2009). Like these bacterial melibiose transporters, MBT1 from *C. graminicola* has 12 predicted transmembrane helices, but it does not share significant sequence similarity with these proteins. MBT1 is rather related to the well-characterized, Mal11p, Mal61p and Agt1p maltose transporters from baker’s yeast (Chang and Michels, 1991; Han et al., 1995), which catalyze the energy-dependent uptake of maltose exclusively together with H⁺ ions (Serrano, 1977; Han et al., 1995).

**MBT1, a Plasma Membrane-Localized, High-Affinity Melibiose Transporter**
After the identification of the *C. graminicola* MBT1 transporter, BLAST searches in publically available protein sequences identified a large number of transporters of exclusively fungal origin. Many of these proteins were deposited as maltose permeases or α-glucoside transporters, however, with the exception of the above-mentioned disaccharide transporters from baker’s yeast hardly any of these transporters have been functionally characterized. One of the identified MBT1-related proteins was the recently published *Metarhizobium* raffinose transporter Mrt1 from *Metarhizium robertsii* (formerly known as *Metarhizium anisopliae*), which enables *M. robertsii* to grow on the disaccharides maltose, sucrose and lactose, on the trisaccharides raffinose and melitzitose, on the tetrasaccharide stachyose, and even on the pentasaccharide verbascose (Fang and Leger, 2010).

Our characterization of the recombinant *C. graminicola* MBT1 protein in baker’s yeast demonstrated that MBT1 catalyzes the uptake of maltose (Fig. 2) with a Km of about 8 mM (Fig. 7). As this Km is in the same order of magnitude as the Km values of the yeast maltose transporters, MBT1 could easily pass for a low affinity maltose transporter. However, competition analyses with other potential substrates immediately showed that maltose is by far not the best substrate of MBT1. Other compounds, preferably α-galactosides such as raffinose, melibiose and galactinol, reduced the uptake of 14C-labeled maltose significantly or inhibited almost completely even at equimolar concentrations (Fig. 3). In further transport studies we could demonstrate that these competitors not only inhibited the uptake of maltose. They were all efficiently imported into *MBT1*-expressing baker’s yeast (Figs. 4 and 5) suggesting that MBT1 is a high affinity α-galactoside transporter with a capacity to transport α-glucosides with lower affinity. This was confirmed when we determined the affinity of MBT1 for raffinose, which turned out to be more than 20-fold higher than its affinity for maltose (Fig. 7B and 7C). From the observation that melibiose is
an even better inhibitor of maltose transport than raffinose (Fig. 3A and 3C) and that more melibiose than raffinose is imported into *MBT1*-expressing baker’s yeast, one can predict that melibiose is the preferred substrate of MBT1 and that the affinity for melibiose is even higher than that for raffinose. Due to the lack of radiolabeled melibiose, however, more detailed analyses could not be performed.

In summary, our data characterize MBT1 as a eukaryotic melibiose transporter that also accepts other α-galactosides, including galactinol and raffinose. α-Glucosides are transported with lower affinity; sucrose is not a substrate of MBT1 (Fig. 2).

The low E-values obtained during our BLAST searches with MBT1 and the phylogenetic analyses presented in Figure 1 demonstrate that MBT1 is not an exception. MBT1 rather seems to be the prototype of a long list of hitherto uncharacterized melibiose transporters from pathogenic fungi, including members of the genera *Magnaporthe, Gibberella, Verticillium, Fusarium* or *Ustilago*.

**MBT1 is an Active, Energy-Dependent Transporter**

Based on its similarity to the yeast maltose H⁺ symporters MBT1 is expected to catalyze an active, energy-dependent transport and to couple this transport to the downhill gradient of H⁺ ions. Although direct proof for such an H⁺ symport mechanism is lacking, as our attempts to express *MBT1* in *Xenopus laevis* oocytes and to directly study the nature of the co-transported ion by patch-clamp analyses failed, it is supported by several lines of evidence: Firstly, MBT1 has its transport optimum at pH 6 and its transport activity decreases steeply at higher pH values (Fig. 6A), which is typical for H⁺ symporters, secondly, the transport by MBT1 is sensitive to CCCP, an uncoupler of transmembrane H⁺ gradients (Fig. 6B,C), and thirdly, all substrates tested were accumulated inside the MBT1-expressing yeast cells to concentrations...
that were significantly higher than the concentrations in the extracellular medium (Fig. 5).

**MBT1 is Essential for the Uptake of Melibiose in *C. graminicola***

Comparative growth analyses of a WT strain of *C. graminicola* and a *C. graminicola* mutant with a disrupted *MBT1* gene (Fig. 9A) on different organic carbon sources demonstrated that MBT1 is essential for the growth of *C. graminicola* on melibiose as sole carbon source (Fig. 9B). In contrast, on all other carbon sources, including raffinose, growth rates of *C. graminicola* WT and mutant strains were identical. This result not only confirmed the melibiose transporter function of MBT1 that had been characterized in baker’s yeast. It also demonstrated that MBT1 is indispensable for melibiose utilization by *C. graminicola*, but not essential for growth on raffinose, another excellent substrate of MBT1. Obviously, a different mechanism such as the direct uptake of raffinose by a transporter similar to the Mrt protein from *M. robertsii* (Fang and Leger, 2010) or the extracellular hydrolysis of raffinose is used for this substrate. In fact, CgAGT4 is a candidate for a putative *C. graminicola* raffinose transporter, as it is closely related to Mrt (69.3% identical amino acids shared between CgAGT4 and Mrt versus 42.4% shared between MBT1 and Mrt) and clusters with Mrt in phylogenetic analyses (Fig. 1).

The observed growth rates of the *C. graminicola* WT strain on melibiose as sole carbon source were slower than the rates on all other carbon sources analyzed (Fig. 9B). Knowing that the *MBT1* gene has a uORF for the 3-aa peptide Met-Val-Thr in its 5’-flanking region at -19 this is not unexpected, as uORFs are known to affect the expression of the following gene (Vilela and McCarthy, 2003) and as the presence of the *MBT1* uORF reduces the transport rates both for maltose (Fig. 2B and 2C) and raffinose (Fig. 4). In baker’s yeast, properties of the transporter such as the sensitivity
to CCCP or the substrate affinity are not affected by the uORF (Figs. 6B,C, 7B and 7C). uORFs were also found in a related but uncharacterized gene from G. clavigera (EFX01607), and a uORF of unknown physiological function has been described in the 5'-flanking region of the distantly related gene for the Hol1p histidinol transporter from baker’s yeast (Wright et al., 1996). This uORF confers translational repression upon HOL1, and disruption of the putative start codon of this uORF resulted in a 5- to 10-fold increase in the steady-state amounts of Hol1p. Although this factor is quite similar to the observed increase in transport rates in MBT1-uORF versus MBT1+uORF yeast cells (Figs. 2 and 4), the regulatory mechanism of the uORF in MBT1 regulation remains elusive and will need to be studied in C. graminicola directly.

When we performed virulence assays with the C. graminicola WT strain, with two Δmtb1 C. graminicola mutants and with a control strain harboring an ectopic insertion of the hygromycin resistance cassette we did not detect any difference (Fig. 9C). Although the abundance of MBT1 mRNA is low compared to the HXT3 mRNA levels at all stages of infection (Fig. 8C), it is comparable to the MBT1 mRNA levels in axenic cultures (Fig. 8A and 8B), where these levels are fully sufficient for the observed growth on melibiose (Fig. 9B). This and the increase in MBT1 expression during the infection (Fig. 8C) suggest that C. graminicola can take up melibiose during its biotrophic and even more during its necrotrophic development. The lack of an obvious phenotype, however, indicates that melibiose and other MBT1 substrates, such as galactinol, represent just a fraction of the organic carbon used by C. graminicola during its pathogenic development. As melibiose is not a prominent sugar in higher plants, this is not unexpected. Nevertheless, our data show that phytopathogenic fungi have developed a multitude of transporters enabling these pathogens to use a wide spectrum of molecules. Quite likely, they also adjusted the
expression of the respective genes to the availability of the different substrates. For the low-level disaccharide melibiose, a uORF might be a way to avoid excess production of MBT1.

MATERIALS AND METHODS

Strains, Growth Conditions and Transformation

C. graminicola strain M2 was used in this study. Axenic C. graminicola cultures were grown in minimal medium containing either 1.5% (w/v) glucose or 1.5% glucose plus 1.5% (w/v) maize cell walls plus 1.5% (v/v) soluble maize leave extract, or 1.5% (w/v) maize cell walls plus 1.5% (v/v) soluble maize leave extract (Krijger et al., 2008). S. cerevisiae strain SEY2102 (Emr et al., 1993) was used for transformation (Gietz et al., 1992). Yeast cells were grown in CAA medium (0.67% yeast nitrogen base w/o amino acids and 1% casaminoacids plus required amino acids) with 2% glucose at 29°C. Protoplasting of oval conidia and transformation of the deletion cassette into protoplasts was performed as described (Werner et al., 2007).

Identification and Cloning of MBT1 Sequences

For the identification of CgAGT genes, BLAST searches (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) were performed with predicted AGT sequences from several fungi, including U. maydis, N. crassa, M. grisea, G. zeae, Neosartorya fischeri, Chaetomium globosum and others against “Colletotrichum graminicola - WGS” sequences provided via the “trace archives” of this WEB-site. Identified shotgun sequences (typical size: about 300 base pairs) were assembled.

The MTB1 cDNA was amplified from total C. graminicola RNA [primers: CgSUC1(fw)voll (5’-GAG AGA GAA TTC GGG AAA TGG TGA CCT GAG C-3’) and CgSUC1(rev)voll (5’-GAG AGA GAA TTC AGA TAT TGT GCT CGA GTC AAG G-3’)]
for \textit{MTB1} (+\ uORF), and CgAGT1c-1fw(voll) (5'-GAG AGA GAA TTC GAT GGC CTC TTC ACA CGA CGG-3') and CgSUC1(rev)voll (5'-GAG AGA GAA TTC AGA TAT TGT GCT CGA GTC AAG G-3') for \textit{MTB1} (-\ uORF)]. Sequenced fragments were cloned into the yeast/\textit{E. coli} shuttle vector NEV-E (Sauer and Stolz, 1994).

\textbf{Transport Measurements}

For transport tests with radiolabelled substrates yeast cells were grown to an $A_{600\text{nm}}$ of 1.0, harvested, washed with 50-mM Na-phosphate buffer pH 5.0 and re-suspended in 10 ml buffer. D-glucose was added to a final concentration of 10 mM to energize the transport. Tests were performed as described (Lingner et al., 2011).

For transport tests with unlabeled substrates, yeast cells were incubated in 50-mM Na-phosphate buffer pH 5.0 with the respective substrate (1 mM) in a rotary shaker at 29°C. After 1 h, cells were harvested, washed 3 times with H$_2$O, incubated for 15 min at 45°C in the vacuum (Speedvac: Fisher Scientific GmbH, Schwerte, Germany), and weighed. Sugars were extracted with 1 ml of 80\% ethanol (1 h, 80°C). Non-soluble material was removed by centrifugation (5-min, 14,000 rpm) and 0.8 ml of the supernatant were desiccated at 45°C in the vacuum (Speedvac). Dry material was solved in 0.25 ml H$_2$O and after a 5-min centrifugation (14,000 rpm, 4°C) 0.1 ml of the supernatant were used for ion chromatography in an ICS-3000 system (Dionex GmbH, Idstein, Germany) with a pulsed amperometric detector (ICS-3000 DC). Due to the high pH of the eluent [500 mM sodium hydroxide in purest water (Millipore GmbH, Schwalbach, Germany)], sugars and sugar alcohols were ionized and separated on a CarboPack MA1 column (4 x 250 mm) connected to a guard column of the same material (4 x 10 mm). The column was equilibrated at a flow rate of 0.4 ml min$^{-1}$; run duration was 80 min. An ATC-1 anion-trap column
between eluent and separation columns removed anionic contaminants. Calibration and quantitative calculation was performed with the Dionex chromeleon software 6.7.

**Targeted Inactivation of MBT1**

The cassette used for targeted deletion of *MBT1* consisted of a 1,001-bp fragment homologous to the left flank of the *MBT1* gene followed by a 2,095-bp fragment containing the hygromycin phosphotransferase (*hph*) gene under the control of the *gpdA* promoter of *Aspergillus nidulans* and a 985-bp fragment homologous to the right flank of *MBT1*. The left and right flank was amplified from genomic DNA of *C. graminicola* using the primers CgAGT1-LB-fw (5'-TAG TAG ACC CAA GCG CAA AG-3'), CgAGT1-LB-fw-n (5'-CAG GCT GGA TAT CAC AAA GG-3'), CgAGT1-LB-unihyg-rev (5'-GTG CAA CTG ACA GTC GTA CA GCG TAA ACG ACG AAT TCT G-3'), CgAGT1-RB-unihyg-fw (5'-GTC TGG AGT CTC ACT AGC TT GTA GAC GTC ACT CGT CAA G-3'), CgAGT1-RB-rev-n (5'-TAG TCG CCC ACC TTG ACA AT-3') and CgAGT1-RB-rev (5'-ACG GTC ATA TAG CCG TCC AT-3'). The *hph* gene was amplified using the vector pPK2 as template and the primers unihyg-F1 (5'-TGT ACG ACT GTC AGT TGC AC T GAC CGG TGC CTG GAT CTT C-3') and unihyg-R1 (5'-AAG CTA GTG AGA CTC CAG AC G GTC GGC ATC TAC TCT ATT CC-3'). Sequence overlaps of the primers with the *hph* gene are underlined. The construction of the complete deletion cassette was done by DJ-PCR as described (Yu et al., 2004).

**RNA Isolation and Quantitative RT-PCR**

RNA isolation and qRT-PCR reactions were performed as described (Lingner et al., 2011). Oligonucleotide sequences used for qRT-PCRs are CgACT-qRT.F1 (5'-TTC TAC GAG CTT CCT GAC GG-3') and CgACT-qRT.R1 (5'-CCG CTC TCA AGA
CCA AGG AC-3’) for CgACTIN, Histon3-5’ (5’-GGA GGT CGG ACT TGA AGT CCT-3’) and Histon3-3’ (5’-CGA GAT CCG TCG CTA CCA GA-3’) for HISTON3, CgAGT1c+1675f (5’-ACC TTC GTC TGG GCC TAT TT-3’) and CgSUC1-rev (5’-AAG ATT TCC ACC TCG GTA CTC TC-3’) for MBT1, UL19(fw) (5’-ATT GTC GCT GGT CGT CTC AT-3’) and UL20(rev) (5’-CAA TGG TGA TGC AGA ACT GG -3’) for CgHXT3.

Quantitative real-time RT-PCRs were performed on a RotorGene 2000 (Corbett Research, Sydney, Australia) with QuantiTect_SYBR_Green PCR Master Mix from Qiagen. Samples were standardized to C. graminicola ACTIN mRNA levels.

DNA Extraction and Genomic Southern Blot Analyses

Genomic DNA of C. graminicola was isolated from vegetative mycelia as described (Döbbeling et al., 1997). For Southern hybridization, Ncol-digested DNA was separated on 0.8% (w/v) TAE-agarose gels, depurinated and blotted onto a positively charged nylon membrane (Hybond-N+; Amersham Pharmacia Biotech) by downward alkaline capillary transfer (Brown, 1999). The alkali-labile DIG-dUTP–labeled hph probe (Roche Diagnostics) was amplified using the pPK2 vector as the template and the primers Hyg-fw (5’-ATC GCT GCG GCC GAT CTT AG-3’) and Hyg-rev (5’-GGT CGG CAT CTA CTC TAT TCC-3’). The labeled MBT1 probe was amplified from genomic DNA using the primers CgAGT1-probe-fw (5’-TAC GGC GAT AAG AAG GAC AG-3’) and CgAGT1-probe-rev (5’-CCA CGT TGA TGC AGA ACT GG -3’). After hybridization of both probes with the blotted DNA, probe detection was performed as recommended by the manufacturer (Roche Diagnostics). The membrane was exposed to ECLX-ray Hyperfilm (Amersham Pharmacia Biotech).

Virulence Assays
Corn (Zea mays cv. Nathan) plants and leaf segments were used to assess virulence of the C. graminicola WT isolate M2, two independent ΔCgmbt1 strains and a transformant carrying an ectopically integrated deletion cassette as described by Münch et al (2011).

**Vegetative Growth Experiments**

To compare utilization of different sugars, the WT isolate and transformants of C. graminicola were grown on minimal agarose medium [1.0 g/l Ca(NO₃)₂, 0.2 g/l KH₂PO₄, 0.25 g/l MgSO₄, 0.054g/l NaCl, 0.01% (w/v) yeast extract, 1.5% (w/v) agarose (biozym, Germany) supplemented with 2% (w/v) one of the sugars glucose, maltose, sucrose, melibiose, raffinose, pectin, starch, cellulose. Aqueous conidial suspensions of C. graminicola were prepared from two weeks old OMA plates, washed twice by centrifugation (4,000 x g, 10 min, 4°C). Subsequently, plates containing different carbon sources were inoculated with a 10 µl droplet of a conidial suspension containing 10⁴ freshly harvested conidia and incubated at 23°C in darkness. Radial growth rates were measured between day three and day eight.

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**LITERATURE CITED**


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Biophys Acta **1354**: 7-12.


FIGRE LEGENDS

Figure 1. Phylogenetic analysis of MBT1 and related transport proteins from fungi
and plants. Phylogenetic tree (maximum likelihood) of MBT1, of CgAGT2 to CgAGT5 protein sequences (accession numbers: FN433110, FN433112, FR667745 and FR667746), of characterized disaccharide [Mal11p from *Saccharomyces cerevisiae* (P53048), SRT1 from *Ustilago maydis* (ACN74541)] or trisaccharide [Mrt from *Metarhizium robertsii* (GQ167043)] transporters from other fungi, of MBT1-related proteins from *Magnaporthe grisea* (EDJ94788), *Grosmannia clavigera* (EFX01607), *Verticillium albo-atrum* (EY19969 and EY23935) and *Gibberella zeae* (XP_380246), and of Arabidopsis sucrose transporters AtSUC1 (CAAA5147), AtSUC2 (CAAA5150), AtSUC3 (CAB92307), AtSUC4 (CAB92308) and AtSUC5 (CAC19851). Bootstrap values of 1000 samplings are given at relevant branches.

**Figure 2.** Sucrose and maltose uptake into baker’s yeast cells expressing the *MBT1* cDNA (± uORF) in sense or antisense orientation. A, Uptake of $^{14}$C-labeled sucrose into *MBT1*-expressing yeast cells (+ uORF = ULY24, closed circles) or into *MBT1*-antisense cells (+ uORF = ULY24as, negative control; open circles) and SRT1 from *U. maydis* in sense orientation (positive control; dotted line). B, Uptake of $^{14}$C-labeled maltose into *MBT1* expressing yeast cells (+ uORF = ULY24, closed circles) or into control cells (+ uORF = ULY24as, open circles). C, Uptake of $^{14}$C-labeled maltose into *MBT1* expressing yeast cells (- uORF = ULY38, closed circles) or into control cells (- uORF = ULY38as, open circles). Note the different lengths of the time axes in B and C. Uptake were performed at the indicated initial substrate concentrations at cell densities of 20 (A and B) or 40 OD$_{600}$ (C).

**Figure 3.** Identification of MBT1 substrates by competition analysis. (A) and (B) show uptake rates of $^{14}$C-labeled maltose into *MBT1*-expressing ULY24 cells (+ uORF) at pH 6.0 in the absence (white bars) or presence of the indicated competitors that were
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Figure 5. Uptake of unlabeled substrates by yeast cells expressing \textit{MTB1} in sense or antisense orientation. Bars show the intracellular concentrations of the indicated substrates in yeast cells expressing \textit{MTB1} in sense (s = ULY38) or antisense orientation (as = ULY38as) after a 1-h incubation in the respective compound (1 mM). In antisense cells, neither melibiose nor raffinose or galactinol could be detected. Trehalose was the only compound found also in antisense cells. The very right bar shows the difference between the trehalose levels in sense and antisense cells (s-as). The dotted line indicates the concentration equilibrium between extracellular and intracellular substrate (n = 3 ± SE).

Figure 6. pH-Dependence of MBT1 and sensitivity to different inhibitors. A, Transport rates for $^{3}$H-raffinose (1 mM) were determined at the indicated pH-values at a cell density of 40 OD$_{600}$ in \textit{MBT1}-expressing ULY24 cells (n = 3; ± SE). B, \textit{MBT1} in ULY24 cells (+ uORF). C, \textit{MTB1} in ULY38 cells (- uORF). Uptake of $^{3}$H-labeled...
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**Figure 7.** Substrate affinities of MBT1 for maltose and raffinose. Uptake rates for 14C-labeled or 3H-labeled substrates were determined at pH 6.0 with the indicated substrate concentrations. A, Michaelis-Menten kinetics of maltose uptake in MBT1-expressing cells (+ uORF = ULY24). B, Michaelis-Menten kinetics of raffinose uptake in MBT1-expressing cells (ULY24). C, Michaelis-Menten kinetics of raffinose uptake in MBT1-expressing cells (- uORF = ULY38). Inserts show double reciprocal (Lineweaver-Burk) plots of the same data (n = 3; ± SE).

**Figure 8.** Quantitative RT-PCR analyses of MBT1 transcript levels. A, MBT1 mRNA abundance in C. graminicola cells grown for 3 d at 23°C in liquid medium in the presence of the indicated additions (glucose = 1.5%; extract = maize cell walls plus soluble extract from maize leaves as described in Materials and Methods). B, MBT1 mRNA abundance in C. graminicola cells grown for 3 d at 23°C in liquid medium supplemented with the indicated α-galactoside (mel = 1.5% melibiose; raf = 1.5% raffinose; w/o = no α-galactoside added). C, MBT1 mRNA levels in C. graminicola-infected maize leaves at 2, 3 or 4 dpi. A, B, and C show HXT3 mRNA levels for comparison (maximum HXT3 levels were set to 100%). Inserts show MBT1 mRNA levels at different resolution (maximum MBT3 levels were set to 100%). Actin (for C) or Histone3 transcript levels (for A and B) served as controls. RT-PCRs were performed on total RNA isolated from healthy or infected maize leaves (maize plus fungal RNA) at the indicated times (3 biological repeats; ± SE).
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