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*). 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 *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 Δ*mbt1* mutants are unable to grow on melibiose, they do not show virulence defects on maize.

In a search for neutral, nonpermeating osmolytes suitable for studies involving the response of suspension-cultured tobacco (*Nicotiana tabacum*) cells to water deficit, Dracup et al. (1986) identified the disaccharide melibiose (α-d-Galp-1,6-d-Glc) 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 (α-d-Galp-1,6-α-d-Glc-1,2-β-d-Fru). Raffinose hydrolysis was mediated by cell wall-bound invertases (α-d-Glc-1,2-β-d-Fru; Roitsch and González, 2004; Vargas et al., 2009), but of the resulting products, only Fru 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* spp.; 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 β-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 (*Arabidopsis thaliana*) plants (Fiehn et al., 2000).

Plasma membrane-localized disaccharide transporters of plants catalyze the uptake of Suc or maltose (α-d-Glc-1,4-α-d-Glc; Sauer, 2007), but proteins transporting raffinose or melibiose have so far not been identified. On the other hand, numerous cell wall-localized α-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 Suc, 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 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 (α-d-Galp-1,6-α-d-Galp-1,6-α-d-Glc-1,2-β-d-Fru) as the inducing compounds, suggesting that these oligosaccharides are secreted to feed rhizosphere symbionts. In fact, a raffinose uptake system (*Metarhizium* raffinose transporter [Mrt]) was identified in the mutualistic soil fungus *Metarhizium robertsi* (Fang and St Leger, 2010) that allows this fungus to grow on different di- and oligosaccharides including Suc, lactose (β-d-
RESULTS

Identification of MBT1 and Sequence Analyses

BLAST searches with α-glucoside transporter (AGT) sequences from baker’s yeast and other fungi in the Na-

Galp-1,4-α-GlcP), raffinose, stachyose, or melizitose (α-D-GlcP-1,3-β-D-Fruf-2,1-α-D-GlcP).

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 (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 (Zea mays) smut, has a plasma membrane-localized Suc transporter, Srt1, that enables this fungus to feed on apoplastic Suc (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 hemi-

biontrophic 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 TRANSPORTER1 (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 Glc 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.
MBT1 is a 611-amino acid protein with 12 predicted transmembrane helices and a deduced molecular mass of 67.62 kD. The comparison of MBT1 genomic (GenBank accession no. FN433107) and cDNA sequences (accession no. 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 no. EYE19969] and MAL61 [accession no. EYE23935]), Grommiana clavigera (uncharacterized protein: accession no. EF01607), Gibberella zae (uncharacterized protein: accession no. XP_380246), or Magnaporthe grisea (uncharacterized protein: accession no. 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 amino acids) 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 three genes. Analyses of the genomic sequences of these genes revealed another interesting feature. In the 5’-untranslated region of the MBT1 gene we detected an upstream open reading frame (uORF) that starts at −19 and encodes a 3-amino acid peptide (Met-Val-Thr). Interestingly, analyses of the 5’-untranslated regions of the MBT1-related genes identified four 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 Suc and maltose. Figure 2A demonstrates that the uptake rates for 14C-labeled Suc (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 U. maydis Suc transporter SRT1 (positive control; Wahl et al., 2010) showed high transport rates for Suc under these conditions, indicating (1) that either Suc is a poor or no substrate for MBT1, (2) that MBT1 is not or only weakly expressed in baker’s yeast, or (3) that MBT1 is not targeted to the baker’s yeast plasma membrane. The two last points could be excluded by the transport analyses with 14C-labeled maltose (1 mM). 14C-Maltose was imported at significantly higher rates into MBT1-expressing cells than into antisense controls (Fig. 2, B and 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 baker’s...
yeast, 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 α-Galactoside Transporter with Preference for Melibiose**

To identify other substrates of MBT1, we tested the effects of candidate substrates on the uptake of 14C-labeled maltose. Figure 3A shows that in the presence of equimolar concentrations of unlabeled putative competitors strong inhibition was observed only with the α-galactosides raffinose, galactinol, and melibiose, with melibiose clearly causing the most pronounced inhibition. Of all other tested substrates, only the α-glucoside trehalose (α-D-Glc-1,1-α-D-Glc) 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 four compounds became even more obvious, and less-pronounced inhibition was also observed with the α-glucosides isomaltose (α-D-Glc-1,6-D-Glc) and isomaltulose (α-D-Glc-1,6-D-Fru).

As the competition analyses shown in Figure 3, A and 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. 3, A and 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 baker’s yeast 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 3H-labeled raffinose (Fig. 4). As for maltose (Fig. 2, B and C) these tests revealed uptake of 3H-raffinose only into MBT1-expressing baker’s yeast cells but not into MBT1-antisense control strains (Fig. 4, A and 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. 2, B and 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 (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

---

**Figure 3.** Identification of MBT1 substrates by competition analysis. A and B show uptake rates of 14C-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 present either at the same concentration (≈1 mM; black bars) or at a 10-fold molar excess (≈10 mM; gray bars). C shows the same experiment performed with ULY38 (~uORF) cells (n = 3; ±ss).
MBT1 into ULY38 cells (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 baker’s yeast 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-m-chlorophenylhydrazine (CCCP; Fig. 6, B and C), an uncoupler of transmembrane H+ gradients, and its sensitivity to the sulfhydryl-group inhibitor p-chloromercuribenzenesulfonic acid (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 >> iso-maltose, 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 than 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. 7, B and 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. 3, B and C).

Figure 4. Transport of raffinose in baker’s yeast cells expressing the MBT1 cDNA (+uORF) in sense (black circles) or antisense orientation (white circles). A, Uptake of 14C-labeled raffinose into MBT1 (+uORF = ULY24) expressing yeast cells or into control cells (MTB1 in antisense orientation = ULY24as). B, Uptake of 3H-labeled raffinose into MBT1 (-uORF = ULY38) expressing yeast cells or into control cells (MTB1 in antisense orientation = ULY38as). Uptake experiments were performed at the indicated initial substrate concentrations at a cell density of 40 OD$_{600}$. FW, Fresh weight.

Figure 5. Uptake of unlabeled substrates by yeast cells expressing MBT1 in sense or antisense orientation. Bars show the intracellular concentrations of the indicated substrates in yeast cells expressing MBT1 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 ($n = 3$ ± s). FW, Fresh weight.
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 (d post infection [dpi]; Fig. 8C). Different MBT1 mRNA levels were observed under all growth conditions (Fig. 8, A–C). However, the abundance of MBT1 mRNA was very low under all conditions analyzed. In semiquantitative reverse transcription (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. 8, A–C; Lingner et al., 2011).

The data presented in Figure 8, A and B show only minor responses of the MBT1 mRNA levels to the...
addition of Glc, extracts, or a-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 (Δmbt1 no. 1 and Δmbt1 no. 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 C. graminicola, of the Δmbt1 number 1 and Δmbt1 number 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 Glc, Suc, 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 Δmbt1 number 1 and Δmbt1 number 2 mutants were unable to grow on melibiose as sole carbon source. In contrast, wild-type C. graminicola and the Ect control grew equally well on melibiose-supplemented medium (Fig. 9B).

When we used the C. graminicola wild-type strain, the Δmbt1 number 1 and Δmbt1 number 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 nonmelanized 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., 1997). These proteins are promiscuous with respect to their cotransported ion (Na+, Li+, or H+) and belong to the so-called galactoside-pentoses-hexuronides family of membrane proteins (Reizer et al., 1994; Poolman et al., 1996; Ganea and Fendler, 2009).
with these proteins. MBT1 is rather related to the well-characterized Mal11p, Mal61p, and Agt1p maltose transporters from baker’s yeast (Cheng 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

---

**Figure 9.** Targeted deletion of MBT1 and characterization of Δmbt1 mutants. A, Strategy of targeted deletion of MBT1 and Southern-blot analyses. The wild-type (WT) strain CgM2 of *C. graminicola* was transformed with a hygromycin resistance cassette flanked by 1 kb each of the 5’- and 3’-flanking regions of MBT1 (top section). Homologous integration of the knockout construct and replacement of MBT1 was indicated by replacement of the approximately 6.1-kb wild-type band by an approximately 4.1-kb fragment (Δmbt1 nos. 1 and 2) in Ncol-digested genomic DNA. Transformants carrying an ectopically integrated knockout construct (Ect) show the approximately 6.1-kb wild-type band plus an additional band corresponding to the randomly integrated knockout cassette (bottom section). B, Radial growth rates of the wild type, gene deletion mutants (Δmbt1 no. 1 and Δmbt1 no. 2), and the transformant with a hygromycin resistance cassette (Ect) on minimal agar supplemented by 2% (w/v) of different carbon sources. Growth rates measured during linear growth rates (3 to 8 dpi) revealed specific growth defects on melibiose (data from three biological replicates; ±SD). C, Virulence assays on detached maize leaves at 4 dpi. Macroscopic observations and quantitative determination of ex planta and in planta differentiated infection structures showed no differences in pathogenic development between the wild-type strain, the mutants Δmbt1 numbers 1 and 2, and the Ect control (data from three biological replicates; ±SD; G, Rate of germination; A, rate of formation of nonmelanized appressoria; M, rate of formation of melanized appressoria; P, penetration rate; nic, noninoculated control).
proteins were deposited as maltose permeases or AGTs, 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 Metarhizium raffinose transporter Mrt1 from M. robertsii (formerly known as Metarhizium anisopliae), which enables M. robertsii to grow on the disaccharides maltose, Suc, and lactose, on the trisaccharides raffinose and melitizitose, on the tetrasaccharide stachyose, and even on the pentasaccharide verbascose (Fang and St 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 $K_m$ of about 8 mM (Fig. 7). As this $K_m$ is in the same order of magnitude as the $K_m$ 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 $\alpha$-galactosides such as raffinose, melibiose, and galactinol, reduced the uptake of $^{14}$C-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 $\alpha$-galactoside transporter with a capacity to transport $\alpha$-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. 7, B and C). From the observation that melibiose is an even better inhibitor of maltose transport than raffinose (Fig. 3, A and C) 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 $\alpha$-galactosides, including galactinol and raffinose, $\alpha$-Glucosides are transported with lower affinity; Suc 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 cotransported ion by patch-clamp analyses failed, it is supported by several lines of evidence: First, 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; second, the transport by MBT1 is sensitive to CCCP, an uncoupler of transmembrane H$^+$ gradients (Fig. 6, B and C); and third, 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 wild-type 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 wild-type 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 St 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 wild-type 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-amino acid 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. 2, B and C) 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. 6 and 7, B and C). 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.

**A Eukaryotic Melibiose Transporter**
(Wright et al., 1996). This uORF confers translational repression upon HOLL, and disruption of the putative start codon of this uORF resulted in a 5- to 10-fold increase in the steady-state amounts of Hollp. 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 wild-type strain, with two Δmbt1 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. 8, A and B), 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 multi-tude 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

Colletotrichum graminicola strain M2 was used in this study. Axenic C. graminicola cultures were grown in minimal medium containing either 1.5% (w/v) Glc or 1.5% Glc plus 1.5% (w/v) soluble maize leaf extract, or 1.5% (w/v) maize cell walls plus 1.5% (v/v) Glc or 1.5% Glc plus 1.5% (w/v) maize cell walls (Corbett Research) with QuantiTect_SYBR_Green PCR master mix from Qiagen. Samples were standardized to 1 µg/mL sodium hydroxide in pure water [Millipore GmbH], sugars and sugar alcohols were ionized and separated on a CarboPack MA1 column (4 × 250 mm) connected to a guard column of the same material (4 × 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 chroameleon 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 GGG CAA AG-3'), CgAGT1-LB-fw-n (5'-CAG GCT GGA TAT CAC AAA GG-3'), CgAGT1-LB-rev (5'-GTC AAG G-3') for the primers unihyg-fw (5'-GTC TGG AGT CTC ACT AGC TTA GTA GAC GTC ACT CGT ACA GTC GTA CAG CCG TAA ACG AAT TCT GCT G-3'), CgAGT1-LB-rev-n (5'-GTC TGG AGT CTC ACT AGC TTA GTA GAC GTC ACT CGT ACA GTC GTA CAG CCG TAA ACG AAT TCT GCT G-3') and with a control strain harboring an ectopic insertion of the hygromycin resistance cassette

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 Ustilago maydis, Neurospora crassa, Magnaporthe grisea, Gibberella zeae, Neosartorya fischeri, Chaetomium globosum, and others against Colletotrichum graminicola-whole genome shotgun sequences provided via the trace archives of this Web site. Identified shotgun sequences (typical size: about 300 bp) were assembled.

The MBT1 cDNA was amplified from total C. graminicola RNA [primers: CgSUC1(fw) (5'-GAG AGA GAA TTC CGG AAA TGA CCA CCT GAG C-3') and CgSUC1(rev) (5'-GAG AGA GAA TTC AGA TAT TGT GCT CGA GTC AAG G-3') for MBT1 (C-gorf), and CgAGT1-fw (5'-GAG AGA GAA TTC AGA TAT TGT GCT CGA GTC AAG G-3') for MBT1 (C-uorf)]. Sequenced fragments were cloned into the yeast Escherichia coli shuttle vector NEB-VE (Sauer and Stolz, 1994).

Transport Measurements

For transport tests with radiolabeled substrates yeast cells were grown to an A₅₀₀ of 1.0, harvested, washed with 50-mL Na-phosphate buffer pH 5.0, and resuspended in 10 mL buffer. n-Glc was added to a final concentration of 10 µM 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-mL Na-phosphate buffer pH 5.0 with the respective substrate (1 µM) in a rotary shaker at 29°C. After 1 h, cells were harvested, washed three times with water, incubated for 15 min at 45°C in the vacuum (Speedvac: Fisher Scientific GmbH), and weighed. Sugars were extracted with 1 mL of 80% ethanol (1 h, 80°C). Nonsoluble 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 water 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) with a pulsed amperometric detector (ICS-3000 DC). Due to the high pH of the eluent (500 mM sodium hydroxide in pure water [Millipore GmbH]), sugars and sugar alcohols were ionized and separated on a CarboPack MA1 column (4 × 250 mm) connected to a guard column of the same material (4 × 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 chroameleon software 6.7.
DNA Extraction and Genomic Southern-Blot Analyses

Genomic DNA of *C. graminicola* was isolated from vegetative mycelia as described (Döbbingel et al., 1997). For Southern hybridization, Ncol-digested genomic DNA was separated on 0.8% (w/v) Tris-acetate-EDTA-agarose gels, depurinated, and blotted onto a positively charged nylon membrane (Hybond-N+; Amersham Pharmacia Biotech) by downward alkaline capillary transfer (Brown, 1999). The alkaline-labile DRC-IUTP-labeled hap6 probe (Roche Diagnostics) was amplified using the pPK2 vector as the template and the primers Hggy-fw (5’-ATC GCT GCC GGC CAT CTG AG-3’ and Hggy-re (5’-GGT CGG CAT CTA CTC TAC TCG CAG-3’). The labeled MBT1 probe was amplified from genomic DNA using the primers CgAGT1-probe-fw (5’-TAC GCC GAT AAG AGA GGC AG-3’) and CgAGT1-probe-re (5’-CCA CGT TGA GCT AGT TG-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 (‘Nathan’) plants and leaf segments were used to assess virulence of the *C. graminicola* wild-type isolate M2, two independent ΔCgm118 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 wild-type 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, 0.054 g/L NaCl, 0.01% (w/v) yeast extract, 1.5% (w/v) agar (biozym)] supplemented with 2% (w/v) one of the sugars Glc, maltose, Suc, melibiose, raffinose, pectin, starch, and cellulose. Aqueous conidial suspensions of *C. graminicola* were prepared from 2-week-old oatmeal agar plates, washed twice by centrifugation (4,000×g). The alkali-labile DIG-dUTP-labeled detection was performed as recommended by the manufacturer (Roche Diagnostics). The labeled MBT1 gene fusion for study of protein localization was isolated from vegetative mycelia as I-digested *Metarhizium robertsii oligosaccharide transporter and facilitates rhizosphere competency in A. Eukaryotic Melibiose Transporter*
Lingner et al.


