Letters

Grass–endophyte interactions: a note on the role of monosaccharide transport in the *Neotyphodium lolii–Lolium perenne* symbiosis

Introduction

Symbiotic mutualistic associations of plants with ecto- and endomycorrhizal and endophytic fungi are very common in natural and agricultural ecosystems; these fungi improve nutrient acquisition and/or tolerance to biotic and abiotic stresses of their hosts (Smith & Gianinazzi-Pearson, 1988; Nehls *et al.*, 2007; Schardl *et al.*, 2007). A major feature of associations between the heterotrophic fungi and autotrophic plants is an exchange of fungus-derived benefits (e.g. phosphorous in ecto- and endomycorrhizal associations, or antiherbivorous alkaloids in endophytic *Neotyphodium*–grass associations) for plant-derived carbohydrates as carbon (C) and energy source for fungal growth and maintenance (Bago *et al.*, 2000; Nehls *et al.*, 2007).

Neotyphodium/Epichloë spp. endophytic fungi occur in 20–30% of cool-season grass species and are of widespread interest to ecological and agricultural research (Schardl, 2001). These obligate symbiotic fungi are an additional C sink and affect host C metabolism and mobilization (Pan & Clay, 2004; Hunt *et al.*, 2005; Rasmussen *et al.*, 2008, 2009). Under stressful conditions, in particular, such as drought and high temperatures, endophyte symbionts have been shown to enhance host photosynthesis and potentially increase total C reserves (Richardson *et al.*, 1993; Marks & Clay, 1996). It has also been shown that endophyte concentrations are reduced in *Lolium perenne* cultivars accumulating high amounts of water-soluble carbohydrates compared with control cultivars (Rasmussen *et al.*, 2007; Liu *et al.*, 2011). These studies indicate that endophytic fungi are important regulators of host carbohydrate metabolism; and that carbohydrate supply by hosts and C utilization by endophytes interact with each other.

Carbon transfer from host plants to fungal symbionts is catalysed by transporter proteins, and several sugar transporters from mutualistic ecto- and endomycorrhizal fungi have been functionally characterized (Schüβler *et al.*, 2006; Nehls *et al.*, 2007; Helber *et al.*, 2011). In general, it has been assumed that simple soluble sugars such as glucose and fructose are the major source of C for symbiotic fungi (Bago *et al.*, 2000); however, some of the isolated sugar transporters have been shown to be able to catalyse the uptake of cell wall sugars such as mannose and xylose as well (Schüβler *et al.*, 2006; Fajardo López *et al.*, 2008; Helber *et al.*, 2011; Doidy *et al.*, 2012).

To provide more information about sugar uptake by endophytic *Neotyphodium/Epichloë* fungi from their hosts, we report here the isolation and functional characterization of a monosaccharide transporter from *Neotyphodium lolii* (MSTN). Initial studies indicated that MSTN preferentially catalyses the uptake of mannose, a monosaccharide mainly found in polymeric cell wall carbohydrates. We therefore hypothesized that *N. lolii* might be able to hydrolyse cell wall carbohydrates. We tested this hypothesis by quantifying the expression of cell wall carbohydrate hydrolysing fungal mannosidase, cellulase and glucanase, as well as sucrose hydrolysing fungal and plant invertases in cultured *N. lolii* mycelia and in ryegrass cultivars and tissues differing in their endogenous sugar content.

Materials and Methods

Isolation and functional characterization of MSTN from *N. lolii*

The total RNA of *N. lolii* (strain Lp19) was isolated from fungal mycelia cultured in liquid potato dextrose medium (BD Difco™, Becton, Dickinson and Company, North Ryde, NSW, Australia), and cDNA libraries were synthesized using the SMART™ RACE cDNA Amplification Kit (Clontech; Norrie Biotech, Auckland, New Zealand). A gene fragment was identified in an in-house endophyte expressed sequence tag (EST) database (AgR Ltd, Hamilton, New Zealand) using a keyword search ('sugar transporter'); sequence analysis confirmed high similarity with known fungal sugar transporters. Full-length *mstN* cDNA and genomic DNA sequences were amplified (for primers see Supporting Information, Table S1; *mstN* GB Acc.# HQ413097.1) and PCR products were sequenced (Allan Wilson DNA Centre, Massey University, Palmerston North, New Zealand). To build a phylogenetic tree, the MSTN protein sequence was analysed using the software programmes BlastP and ClustalW. A sequence identity dendrogram of fungal sugar transporters (Fig. S1) was obtained using NCBI Blast pairwise alignments by the neighbour-joining method.

A construct containing *mstN* was heterologously expressed in a yeast (*Saccharomyces cerevisiae*) mutant strain (EBY.VW4000) devoid of any monosaccharide transporters (MSTs; Thompson *et al.*, 1998; Schüβler *et al.*, 2006). The mutant yeast strain is unable to take up and grow on hexoses, but it can be cultivated on maltose-containing media (Wieczorke *et al.*, 1999). Initial growth rates of yeast hxt-null recombinants complemented with *mstN* and empty-vector controls were determined in YNB (yeast

nitrogen base) media supplemented with 0.1% of various hexoses. After inoculation, subsamples were collected during a time course (0–171 h) and cell concentrations (OD_{600}) measured using a microplate reader (VERSAmax; Labspec, Hamilton, New Zealand). Least significant differences (LSD) were calculated for separating treatment differences in yeast cell growth.

Uptake experiments using either 1 mM D-[U-^{14}C] mannose (Amersham CFB26, specific radioactivity 111 kBq μmol^{-1}) or D-[U-^{14}C] glucose (Amersham CFB96, specific radioactivity 9435 kBq μmol^{-1}) were performed according to Schüßler *et al.* (2006) and Helber *et al.* (2011). Radioactivity was measured with a liquid scintillation counter (Wallac μBeta Trilux 1450 LSC & Luminescence counter; Perkin Elmer, Melbourne, Victoria, Australia) after addition of a scintillation cocktail (Starscint; Perkin Elmer). K_m values were estimated using the Michaelis–Menten equation ($V = (V_{max}[S])/(K_m + [S])$) with V, ^{14}C-hexose uptake rate and [S], concentration of glucose (or mannose). The pH optimum of D-[U-^{14}C] glucose uptake of *mstN*-expressing yeast cells was analysed in 100 mM potassium phosphate buffer adjusted to pH values 5.0, 6.0, 7.0 and 8.0. To test proton dependency of sugar uptake, yeast cells expressing *mstN* were preincubated for 2 min with 100 μM of the transmembrane proton gradient uncoupler carbonylcyanide *m*-chlorophenyl hydrazone (CCCP) before addition of D-[U-^{14}C] glucose. For all experiments, three independent *mstN*-expressing yeast transformants were used as replication.

Transcript abundance of *mstN*, fungal cell wall hydrolases and invertases in culture and *in planta*

In vitro cultured *N. lolii* (strain Lp19) mycelia from agar plugs were propagated in liquid potato dextrose broth for 2 wk. Washed mycelia were subsequently incubated for 4 d at 26°C in sterile H_2O-based D-glucose, D-mannose and D-fructose solutions. Forty different endophyte-free or *N. lolii* (Lp19)-infected genotypes from two *L. perenne* L. cultivars, the high sugar grass 'AberDove' and a normal sugar grass 'Fennema' (Parsons *et al.*, 2004) were grown in a controlled environment chamber as described previously (Liu *et al.*, 2011). Mycelia, leaf blades and pseudostems were immediately frozen and ground in liquid nitrogen, and then stored at −80°C for RNA isolation.

Total RNA was isolated and reverse-transcribed using the SuperScript®VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative PCR was performed as previously described (Rasmussen *et al.*, 2007). Primers (Table S1) for quantifying the expression of *N. lolii* and *L. perenne* genes were designed for β-1,6-glucanase (Bryant *et al.*, 2007), cellulase, α-mannosidase, fungal invertase, and vacuolar, cell wall and cytosolic plant invertases. Sequences for mannosidase and fungal invertase were obtained from the *Epichlöe festucae* genome database (*Epichloë_festucae*_2368-scaffolds 2011-02 database; http://www.endophyte.uky.edu/). For accession and gene contig numbers, see Table S1. *N. lolii* gene expression data were normalized against the expression of β-tubulin (Bryant *et al.*, 2007); *L. perenne* gene expression data were normalized against the expression of the protein elongation factor Lp*eEFα* (Martin *et al.*, 2008). The transcript abundances are expressed as the ratio of target gene copies to housekeeping gene copies; data were statistically analysed by ANOVA.

Results and Discussion

Based on sequence similarity, conserved motifs and predicted membrane topology, the sugar transporter isolated from *N. lolii* is classified as an MST of the sugar porter family included in the major facilitator superfamily (Notes S1). The MSTN amino acid sequence is highly similar to many putative MSTs from Ascomycetes (Fig. S1), with an 81% amino acid sequence similarity to the hexose transporter CgHXT3 from the hemibiotrophic maize pathogen *Colletotrichum graminicola* (Lingner *et al.*, 2011), but relatively distant from sugar transporters from the ectomycorrhizal Basidiomycetes *Laccaria bicolor* (CAQ53118; 46%) and *Amanita muscaria* (CAB06078; 44%). *N. lolii* is the asexual derivative of *E. festucae* and is nearly indistinguishable from it at the genetic level (Schardl *et al.*, 1994; Schardl, 2001). Alignment of the *mstN* genomic nucleotide sequence with genes in the *E. festucae* genome database (*Epichloë_festucae*_2368-scaffolds 2011-02; http://www.endophyte.uky.edu/) resulted in the identification of EfM2.114080_A061106_1361_3407_5576_Alt0_PolyA (Supercontig 42:67179.688134), which was highly similar to *mstN* (99% identity). Only one sequence with high similarity to *mstN* was identified in the *E. festucae* database, suggesting that *mstN* is a single copy gene in *Neotyphodium/Epichloë* spp. A keyword search ('sugar transporter', 'sucrose transporter') resulted in several hits to putative other sugar/sucrose transporters, but none of these sequences was homologous to *mstN* (data not shown).

The monosaccharide transporter from *Neotyphodium lolii* complemented the sugar uptake deficiency in a yeast strain devoid of all MSTs (Wieczorke *et al.*, 1999), and initial growth rate assays (Fig. 1a) as well as kinetic studies (Fig. 1b) indicated that MSTN has a higher affinity for mannose (K_m = 0.47 mM) than for glucose (K_m = 5.77 mM). Competition assays with ^{14}C-glucose (Fig. 1c) showed that, at high sugar concentrations, glucose was a better competitor than mannose, while the cell wall sugars galactose and xylose were intermediate competitors. Neither fructose nor the sugar alcohols mannitol and myo-inositol competed with uptake of glucose. The optimum pH for glucose uptake was 7.0 (Fig. 1d), and, like many other MSTs, MSTN was inhibited by CCCP (Fig. 1e), indicating it is an energy-dependent H^+-symporter. These results demonstrate that MSTN has the capacity to catalyse the transport of monosaccharides, including the cell wall sugars mannose and xylose. At this stage we cannot exclude the possibility that MSTN is also able to catalyse transport of sucrose, which would require the analysis of MSTN in an invertase deficient yeast strain.

Expression of *mstN* is regulated by available sugar concentrations

Differences in *mstN* transcript abundance were associated with soluble carbohydrate concentrations both in culture and *in planta*, showing a lower expression at high carbohydrate

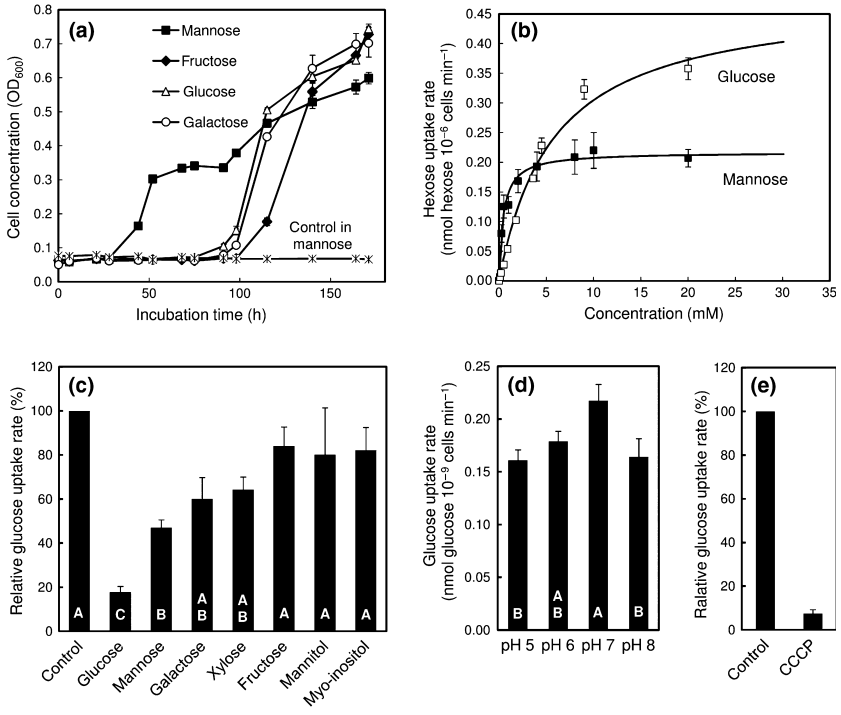

Fig. 1 Functional characterization of monosaccharide transporter from *Neotyphodium lolii* (MSTN) in complemented yeast hxt-null mutants. (a) Initial growth curves in 0.1% hexose supplied media. Squares, D-mannose; diamonds, D-fructose; triangles, D-glucose; circles, D-galactose. Line with crosses, empty vector controls in D-mannose. (b) Uptake kinetics for ^{14}C-glucose and ^{14}C-mannose. (c) Substrate competition of various sugars (3.5 mM) with ^{14}C-glucose (1 mM). (d) Uptake of ^{14}C-glucose at different pH values. (e) Uptake of ^{14}C-glucose in the presence of the protonophore CCCP. Error bars indicate ± SE of three replicates. Different letters in (c and d) denote means that are significantly different.

concentrations. In the absence of any carbohydrate substrates, *mstN* was highly expressed in cultured *N. lolii* mycelia, while addition of hexoses resulted in a strong down-regulation of *mstN* expression (Fig. 2a; $F_{3,11} = 25.29$, $P < 0.001$ for glucose; $F_{3,11} = 5.16$, $P < 0.05$ for mannose; $F_{3,11} = 44.72$, $P < 0.001$ for fructose), comparable to expression patterns seen for hexose transporters from the ectomycorrhizal fungi *Tuber borchii* and *L. bicolor* (Polidori *et al.*, 2007; Fajardo López *et al.*, 2008), as well as for CgHXT3 from *C. graminicola* (Lingner *et al.*, 2011). Expression of β-tubulin was not different in mycelia from *N. lolii* grown in hexose-free water compared with hexose containing water as indicated by the Ct values (data not shown).

In endophyte-infected plant blades and pseudostems, the relative transcript abundance of *mstN* were significantly lower in the high sugar cv 'AberDove' than in the control cv 'Fennema' (in blades: $F_{1,77} = 8.84$, $P < 0.01$; in pseudostems: $F_{1,77} = 4.18$, $P < 0.05$); relative expression was also reduced in pseudostems compared with blades ($F_{1,155} = 9.47$, $P < 0.01$; Fig. 2b). The total water-soluble carbohydrate concentrations were significantly higher in 'AberDove' than in the control 'Fennema'; they were also significantly higher in pseudostems than in blades (Notes S2). It is possible that *mstN* is primarily important for sugar uptake at low carbohydrate availability (hence its relatively high expression under these conditions), while at high availability, other sugar uptake mechanisms might be activated, as was reported for *Acremonium typhinum* (syn. *Neotyphodium typhinum*) sugar uptake (Lam *et al.*, 1994).

Transcript profiles of carbohydrate hydrolases in culture and *in planta*

The prevalent hypothesis for grass–*Neotyphodium*/*Epichloë* associations is that the endophyte takes up glucose and fructose derived from hydrolysis of sucrose by invertases in the apoplastic space in which the fungal hyphae reside (Thrower & Lewis, 1973; Lam *et al.*, 1994). In culture studies by Lam *et al.* (1994), it was shown that *A. typhinum* invertase activity was induced by sucrose and the authors suggested that the majority of the high invertase activity seen in infected plant tissues is the result of fungal invertases. In support of this, we show here that relative expression of a putative fungal invertase identified in the *E. festucae* genome was higher *in planta* than in culture ($F_{2,29} = 26.67$, $P < 0.0001$; Fig. 2c). We also show that two of the three host plant invertases tested, a vacuolar and an apoplastic/cell wall invertase were significantly down-regulated in *N. lolii*-infected plants compared with uninfected plants ($F_{1,39} = 19.15$, $P < 0.0001$ for vacuolar invertase; $F_{1,39} = 20.49$, $P < 0.0001$ for cell wall invertase; Fig. 2d), while a cytosolic invertase was not affected. These results possibly indicate that the infected host plant can reduce sucrose hydrolysis (and thereby hexose availability in the apoplastic space), while the fungus has the ability to 'override' plant control of sucrose hydrolysis. However, as the genome of *L. perenne* has not been sequenced yet, we cannot exclude the presence of additional host invertases which might be regulated in a different manner.

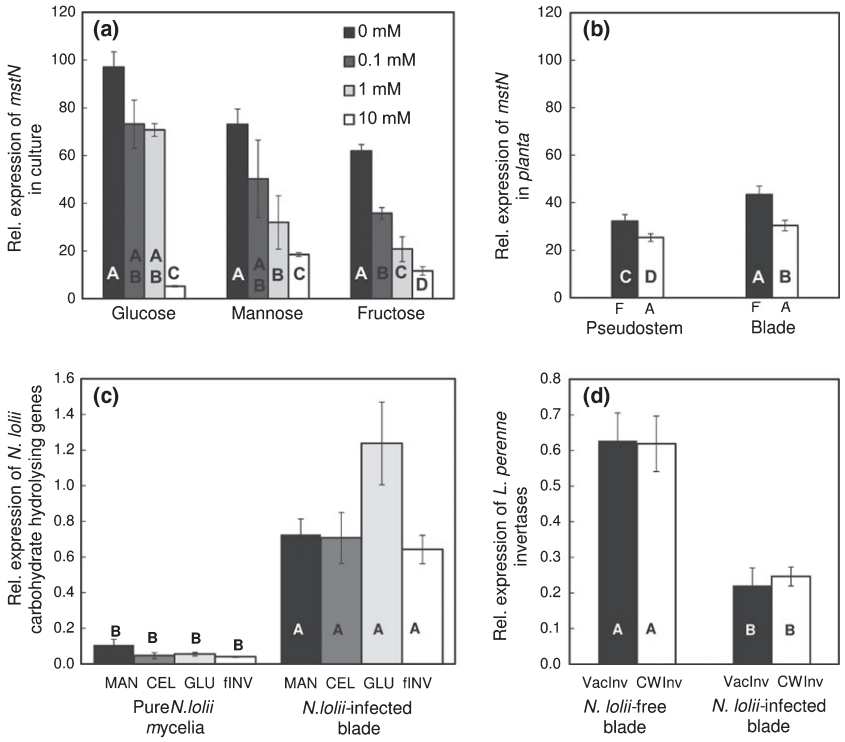

Fig. 2 Expression analysis of *mstN* and carbohydrate hydrolases in culture and *in planta*. (a, b) Expression of *mstN* in cultured mycelia of *Neotyphodium lolii* supplied with various hexoses (a) (black bars, 0 mM; dark grey bars, 0.1 mM; light grey bars, 1 mM; white bars, 10 mM hexose), and infected perennial ryegrass (*Lolium perenne*) pseudostems and blades (b) (black bars, Fennema; white bars, AberDove). (c, d) Expression of fungal α-mannosidase (MAN), cellulase (CEL), β-1,6-glucanase (GLU; data scale reduced 10-fold) and invertase (INV) in cultured fungal mycelia (left-hand bars) and in infected *Lolium perenne* plants (right-hand bars) (c), and *L. perenne* specific vacuolar invertase (vINV, black bars) and apoplastic/cell wall invertase (cwINV, white bars) in uninfected (left-hand bars) and infected (right-hand bars) plants (d). Different letters denote means that are significantly different. Vertical bars indicate ± SE.

Based on the uptake characteristics of MSTN, particularly its high affinity for the cell wall carbohydrate mannose, we hypothesized that *N. lolii* might be able to hydrolyse polymeric plant cell wall carbohydrates as a supplementary source of C. The analysis of transcript abundance of fungal genes coding for putative cell wall carbohydrate hydrolases showed that relative expression of α-mannosidase, cellulase and β-1,6-glucanase (Bryant *et al.*, 2007) was significantly higher *in planta* than in culture ($F_{2,29}$ = 15.63, $P < 0.0001$ for mannosidase; $F_{2,29}$ = 6.36, $P < 0.01$ for cellulase; $F_{2,29}$ = 8.126, $P < 0.005$ for glucanase; Fig. 2c), which might suggest that *N. lolii* has the potential to hydrolyse cell wall carbohydrates. In this context it should be noted that a metabolite-profiling study demonstrated that *N. lolii*-infected *L. perenne* plants had reduced amounts of neutral detergent fibres, congruent with a reduction in cell wall hemicelluloses caused by carbohydrate hydrolysis (Rasmussen *et al.*, 2008). In support of this, a recent transcriptome study comparing an *E. festucae* mutant with a wildtype strain *in planta* revealed that the disruption of a mitogen-activated kinase (*sakA*) resulted in a switch from mutualism to pathogenesis (Eaton *et al.*, 2010). Proliferative branched growth of the fungus was accompanied by an up-regulation of fungal cell wall hydrolases. Even though electron micrographs do not show any major damage on cell walls of infected plants (Christensen *et al.*, 2008), it is possible that cell wall degradation is only limited and localized, as is seen in biotrophic fungal infections (Münch *et al.*, 2008).

Concluding remarks

Carbohydrate exchange between host plants and symbiotic fungi is critical for a successful mutualistic association, and the present study contributes some new information on sugar transfer from grass hosts to endophytic *Neotyphodium* fungi. The apparent high affinity of MSTN for mannose and the high expression of putative endophytic cell wall hydrolases *in planta* present additional supportive evidence for the hypothesis of limited cell wall carbohydrate utilization in symbiotic plant–fungal associations, clearly calling for further investigations.

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Supporting Information

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

Fig. S1 Sequence identity dendrogram of the MSTN protein and sugar transporters from various fungi.

Table S1 Primer sequences used in this study for gene isolation and RT-qPCR

Notes S1 Sequence analysis of MSTN.

Notes S2 Water-soluble carbohydrate concentrations in AberDove compared with Fennema and blades compared with pseudostems.

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Key words: endophytic fungi, *Epichloë* spp., high sugar grass, *Lolium perenne*, *Neotyphodium lolii*, plant–fungal interactions, sugar transport.