Sugar Exchanges in Arbuscular Mycorrhiza: RiMST5 and RiMST6, two Novel *Rhizophagus irregularis* Monosaccharide Transporters, Are Involved in Both Sugar Uptake from the Soil and from the Plant Partner

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Abstract

Arbuscular mycorrhizal (AM) fungi are associated with about 80% of land plants. AM fungi provide inorganic nutrients to plants and in return up to 20% of the plant-fixed CO₂ is transferred to the fungal symbionts. Since AM fungi are obligate biotrophs, unraveling how

sugars are provided to the fungus partner is a key for understanding the functioning of the symbiosis. In this study, we identified two new monosaccharide transporters from *Rhizophagus irregularis* (RiMST5 and RiMST6) that we characterized as functional high affinity monosaccharide transporters. RiMST6 was characterized as a glucose specific, high affinity H⁺ co-transporter. We provide experimental support for a primary role of both RiMST5 and RiMST6 in sugar uptake directly from the soil. The expression patterns of *RiMSTs* in response to partial light deprivation and to interaction with different host plants were investigated. Expression of genes coding for RiMSTs was transiently enhanced after 48h of shading and was unambiguously dependent on the host plant species. These results cast doubt on the 'fair trade' principle under carbon-limiting conditions. Therefore, in light of these findings, the possible mechanisms involved in the modulation between mutualism and parasitism in plant-AM fungus interactions are discussed.

1. Introduction

Arbuscular mycorrhiza (AM) is a widespread mutualistic symbiosis system that involves most of land plants, including agriculturally relevant species, and plays an important ecological role. The microbial partners of this symbiosis are AM fungi (AMF) that belong to the Glomeromycota phylum and are multinucleate obligate biotrophs. This symbiotic relationship is based on a nutrient “fair-trade” between fungus and host plant (Kiers et al., 2011). Several mineral nutrients can barely be accessible to plant roots, especially phosphate due to its low mobility and thus its poor availability in the soil (Casieri et al., 2013). But these nutrients are efficiently taken up by the fungal extraradical mycelium (ERM) that develop through the soil, and further transported to the biotrophic interfaces where they are taken up by the host. In return, the photosynthetic partner transfers carbohydrates to the fungal structures that develop within the root cortex. The carbon transfer from the plant to the fungus was first demonstrated by Ho

and Trappe (1973), who showed that ^{14}C accumulates in AM fungal spores derived from plants exposed to $^{14}\text{CO}_2$. However, in which form carbon is given to the fungus and where precisely the transfer takes place long remained a controversial subject (Smith and Smith, 1990). Isotopic labeling and nuclear magnetic resonance spectroscopy using AM-colonized roots showed hexoses, *i.e.* glucose and to a lesser extent fructose, being taken up by the intra-radical mycelium (Solaimanand and Saito, 1997 and Pfeffer et al., 1999). Exchanged nutrients have to go through several membrane barriers at the apoplastic interface before being assimilated by the partner's cells (Casieri et al., 2013), and proton-ATPase activity on the two membranes of the symbiotic interface argues for an active membrane transport mechanism (Krajinski et al., 2014). Nowadays, the site for photosynthate exchange between mycorrhizal symbionts is commonly accepted to be at the arbuscular interface, as demonstrated for phosphate transport (Pumplin and Harrison, 2009). Nevertheless, some authors also suggest that intercellular hyphae could be an important C-exchange site (Helber et al., 2011). Among glomeromycotan fungi, the first symbiosis-related glucose transporter was identified in *Geosiphon pyriformis* in a unique symbiotic interaction with *Nostoc punctiforme*. Thanks to this model, *GpMST1* was isolated (Schüßler et al., 2006, 2007), and characterized as an H^+ -glucose transporter with a K_m of 1.2 mM and highest affinity for glucose and mannose. Information based on this model and the available EST database recently led to the isolation of three sequences with high similarity to genes encoding monosaccharide transporters (MSTs: *RiMST2*, *RiMST3* and *RiMST4*) from the glomeromycotan model fungus *Rhizophagus irregularis* (Helber et al., 2011). Among them, *RiMST2* was described as a high-affinity MST that plays a major role in sugar uptake in the AMF *R. irregularis*. *RiMST2* has a broad substrate spectrum, with highest affinity for glucose. Transcripts coding for *RiMST2* accumulate in arbuscules and intercellular hyphae. Interestingly, although *RiMST2* expression is almost undetectable in the ERM, it is specifically induced following addition of xylose, suggesting that xylose could be the signal that triggers *RiMST2*

expression *in planta*. These results support the hypothesis that AMF can take up sugars not only from the plant but also directly from the soil (Helber et al., 2011). However, mechanisms underlying sugar uptake by fungi and its regulation still remain largely unknown, especially at the arbusculated cell level in the mycorrhizal association.

It is now well established that yeast cells modulate their glucose transporters for available glucose to ensure its efficient uptake and utilization (Kim et al., 2013). Interestingly, work on ectomycorrhizal fungi also provided evidence for different regulatory systems combined with different localizations for MSTs. Some *MSTs* are induced by the extracellular monosaccharide concentration and putatively responsible for glucose uptake at the plant–fungus interface (*e.g.*: *Amanita muscaria* *MST2*; Nehls, 2004) while others are activated during carbohydrate starvation of fungal hyphae (*e.g.* *Tuber borchii* *HXT1*; Polidori et al., 2007) and are probably involved in supplying sugar to the soil-growing mycelium. Such regulatory mechanisms controlling monosaccharide uptake are likely to occur in AM symbiosis but this occurrence is still debated and remains to be deciphered.

Investigating the regulation of monosaccharide uptake by the fungus *R. irregularis* first requires identifying and characterizing its respective sugar transporters. By using the data from the *R. irregularis* DAOM 197198 sequencing project (Tisserant et al., 2012), we identified two new full-length sequences (*RiMST5* and *RiMST6*) corresponding to putative MSTs that we characterized as glucose transporters.

In the current study, we provide experimental support for a primary role of both transporters in sugar uptake directly from the soil. We also report for the first time, that both fungal monosaccharide transporters expression profiles and localizations vary with the host plant, thereby suggesting a versatile role for MSTs, as dependent upon plant investment towards symbiotic interaction (Kiers et al., 2011 and Walder et al., 2012).

2. Materials and methods

2.1. Biological material and growth conditions

The arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* (DAOM197-198), formerly known as *Glomus intraradices*, was provided by INRA (Dijon). It was first produced *in vitro* and used for further inoculation of *Allium porum* L; the final inoculum was obtained as a mixture of soil, mycorrhizal roots, hyphae and spores. Seeds of *Medicago truncatula* Gaertn. cv. Jemalong, line J5 were surface-sterilized for 6 min in 98% sulfuric acid, rinsed 3 times (1 min each) in sterile water, placed for 3 min in 100% ethanol, and then rinsed as described before. They were germinated for 3 days on 0.7% Bactogar (Difco Laboratories, Detroit) at 25°C in the dark. Seedlings were transplanted into 75-mL pots containing sterilized (autoclaved 2x6h at 180°C) sand. Soil-based inoculums of *R. irregularis* were added to the soil in a thin layer for “mycorrhizal” plants. Plants were grown under constant conditions (355 $\mu\text{E}/\text{m}^2/\text{s}$, 24/21°C, 16/8h day/night and 70% humidity) and received 5 mL of Long Ashton solution without phosphate twice a week. They were harvested 28 dpi; part of the roots was washed and immediately stored in liquid nitrogen for RNA extraction.

Pure spores of *R. irregularis* were provided by the company Symplanta (Symbiosis and Plant-Microbe Association Research laboratory, Dr. Arthur Schüßler) as a concentrate of 20,000 spores in 10 mL of gellan-gum gel. The suspension was sieved (63 μm), washed under running tap water and spores were collected. Batches of approximately 2,000 spores were germinated for 10 days in 450 nM apigenin at 25°C in 2% CO₂, collected by centrifugation and immediately used for RNA extraction.

2.2. *In silico* analysis

Putative fungal transporter sequences from *Rhizophagus irregularis* DAOM197198 were retrieved from the available preliminary genomic data (<http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Gloir1>) by using key word searches and BLAST programs with

sequences from the *Geosiphon pyriformis* hexose transporter (*GpMST1*), the 15 members of the *Laccaria bicolor* sugar transporter family (López et al., 2008), the fructose transporter *BcFRT1* (*Botrytis cinerea*, GenBank CCD42948) and the sucrose transporter *SpSUT1* (*Schizosaccharomyces pombe*, GenBank CAB16264). A list of about ten sequences of putative sugar transporters was recorded. As the *R. irregularis* genome presents a very low GC content (~30%), six candidates with a high GC% and a BLAST result consistent with bacterial transporters were considered to originate from *Aspergillus* sp. contamination and were withdrawn from the analysis (Tisserant et al., 2012). It turned out that one of the candidates had just been published (*RiMST4*; Helber et al., 2011), so only the tree remaining candidates were retained for further analysis. Finally, only two sequences could be fully amplified and used for transporters characterization (Table S2). The expression profile of *R. irregularis* MST candidates was obtained from the *R. irregularis* expression microarray NimbleGen chip (Tisserant et al., 2012).

2.3. Phylogenetic analysis

Amino acid sequences were imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 5.1 (Tamura et al., 2007) and aligned with MUSCLE. Phylogenetic trees were constructed using the Maximum parsimony method (Tamura et al., 2007). Heuristic tree searches were performed using 1,000 random sequence additions and the Subtree-Pruning-Regrafting (SPR) algorithm with random sequence analysis. Characterized and published proteins were named according to their latest name; accession numbers were used for proteins that had not been characterized yet.

2.4. Cloning of candidate transporters

The predicted coding sequences corresponding to *RiMST5* (1,730 bp) and *RiMST6* (1,495 bp) were amplified from colonized root cDNA generated by PCR using Phusion High-Fidelity

DNA Polymerase and the following primers: *RiMST5_F* (5'-**gggacaagtttgtacaaaaagcaggct**atggaagaagaggaagaattagaac-3'), *RiMST5_R* (5'-**gggacaagtttgtacaaaaagcaggct**atggaagaagaggaagaattagaac-3'), *RiMST6_F* (5'-**gggacaagtttgtacaagaaagctgggt**tataactaaagagtaataagtaatgcc-3'), *RiMST6_R* (5'-**gggacaagtttgtacaagaaagctgggt**atggaagaatccctttcaaaaga-3'). The sequences reported in this paper can be accessed at GenBank (<http://www.ncbi.nlm.nih.gov/>) under the following accession numbers: JN255789 (*RiMST5*) and JN255790 (*RiMST6*). The amplification products, flanked with *attB* sequences, were cloned into the donor vector (pDONR221, Invitrogen) by recombination with *attP* sequences by means of the Gateway® technology using BP Clonase™ II enzyme. The reaction was used to transform TOP10 competent *Escherichia coli* cells. The bacteria containing entry clones were selected and positive plasmids were extracted by plasmid preparation (NucleoSpin® Plasmid Miniprep). After checking the correct integration of the two *RiMST* genes flanked by *attL* sequences into the entry plasmid, recombination with the destination yeast pDRf1-GW (Loqué et al., 2007) vectors containing *attR* sequences was performed using LR Clonase™ II enzyme. *E. coli* cells containing expression clones were selected on LB ampicillin medium and checked by bacterial colony PCR. The PCR products were sequenced.

2.5. Expression analysis

Total RNA extraction was performed with the RNeasy Plant Mini kit (Qiagen, Darmstadt, Germany) on 100 mg of frozen roots of plants inoculated with *R. irregularis* DAOM 197198 and harvested at different stages. In the same way, RNA was extracted from 2,000 germinated spores of *R. irregularis* DAOM and from the ERM. To remove contaminating genomic DNA, the samples were treated with DNase I (Qiagen), as recommended by the manufacturer. RNA quantity and quality were assessed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Willmington, DE). One µg of total RNA was annealed to oligo(dT) primers

(Promega, Madison, WI, USA) and reverse-transcribed using ImpromII Reverse from Promega. Quantitative real-time PCR reactions were performed in a final volume of 15 μ L using Absolute SYBR green ROX Mix (Thermo Scientific), 70 nM gene-specific primers and 2 μ L of cDNA template in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The reaction conditions were set as 95°C for 15 min, and 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. Three biological and three technical replicates were performed. All data were analyzed using SDS 2.3 software (Applied Biosystems). Values were normalized to the constitutively expressed Translation Elongation Factor (*RiTEF*).

2.6. Yeast transformation

The yeast strain EBY.VW4000 (*Mata leu2-3 112 ura3-52 his3- Δ 1 trp1-289 MAL2-8c SUC2 Δ hxt1-17 gal2 Δ stl1 Δ ::loxP agt1 Δ ::loxP Δ mph2 Δ mph*; (Wieczorke et al., 1999) was transformed with pDRf1-GW vectors harboring the *RiMST5* and *RiMST6* cDNA sequences. The pDRf1-GW empty vector was used as a negative control. Yeast transformants were selected first on synthetic maltose medium (SM ura-) consisting of 0.67% (wt/vol) yeast nitrogen base without amino acids supplemented with yeast synthetic dropout medium (Sigma-Aldrich Inc., St. Louis, MO) without uracil, to which were added 2% of maltose and 2% (wt/vol) agar. Yeast strain EBY.VW4000 complementation tests were performed on synthetic media without uracil to which were added different concentrations of D-glucose (SD ura- with 22, 50 or 150 mM)

2.7. Transport measurements

For *Saccharomyces cerevisiae* uptake studies, yeast cells were grown on SM ura- till the logarithmic phase. They were harvested at an OD₆₀₀ of 0.5-0.6, washed twice in sterilized milliQ water, and resuspended in NaPi buffer (0.6 M sorbitol, 50 mM potassium phosphate, at the desired pH) to a final OD₆₀₀ of 5. Prior to the uptake measurements, the cells (100 μ L) were energized with 5 μ L of 10 mM maltose and incubated for 5 min at 30 °C. To start the reaction, 100 μ L of the cell suspension were added to 100 μ L of the same buffer containing at least 10.7

kBq of [^{14}C]Glu and unlabeled glucose at the concentrations used in the experiments. Aliquots of 50 μL were sampled after 60, 120, 180 and 240 sec, transferred into 4 mL of ice-cold NaPi buffer. The cells were vacuum-collected onto GF/C microfiber filters (Whatman) and washed twice with 5 mL of NaPi buffer. Monosaccharide uptake was determined by liquid scintillation spectrometry in a Tri-Carb 2100TR Liquid Scintillation Analyzer (Packard).

The influence of plasma membrane energization on the uptake rate of ^{14}C -glucose was analyzed by incubating the yeast cells for 5 min in the presence of 100 mM glucose (control) or with glucose and 0.1 mM 2,4-dinitrophenol (DNP), 0.1 mM diethylstilbestrol (DES), 0.1 mM carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), or 0.1 mM vanadate. Transport measurements were repeated independently and represent the mean of at least three experiments.

2.8. Effect of AM association with different host plants on MST expression levels

Further experiments were performed with sorghum (*S. bicolor* (L.) Moench cv Pant-5), *Populus trichocarpa* (clone INRA 101-74) and *Medicago truncatula*. *P. trichocarpa* cuttings (propagation by cuttings of 1-cm pieces under sterile conditions) were placed in plastic containers containing 150 mL of Murashige and Skoog (MS) agar and then transferred into a growth chamber with a 16-h-light/8-h-dark cycle at 22°C for one month. *S. bicolor* and *M. truncatula* seeds were surface-sterilized (10 min in 5% NaClO), rinsed with sterile deionized water several times and sowed on autoclaved sand (121 °C twice for 25 min) at 25°C for 24h and then grown in the dark at room temperature for 72h. To establish AM symbiosis, *S. bicolor* and *M. truncatula* seedlings and well-rooted poplar plantlets were individually inoculated (with *R. irregularis* DAOM197198) into compartmented microcosms; one plant and one hyphal compartment were connected, but separated by two 21- μm nylon meshes and an air gap in-between (Koegel et al., 2013). The two compartments were filled with an autoclaved mixture of clinoptinolite (natural zeolite, particle size 2.5–5 mm) and sand (1:1 w/w). About 100 spores

were added to the mixture. For the non-mycorrhized plants, the same amount of autoclaved inoculum was added to the mixture. To correct for possible differences in microbial communities, each pot received one mL of filtered washing of AM fungal inoculum (Walder et al., 2012). Plants ($n = 3$ per treatment) were grown in a greenhouse with day-night temperatures of 28 and 15°C, respectively. They were watered twice a week throughout the whole experiment. From the first week on, 8 mL of modified Hoagland solution were applied weekly (Walder et al., 2012). Roots from the plant compartment and mycelium from the fungal compartment were harvested from each compartmented system three months after inoculation and immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

2.9. Effect of shading on fungal MST expression

Mycorrhizal *S. bicolor* plants used for light stress conditions were inoculated with the fungal strain *R. irregularis* and grown for three months in the greenhouse as described above, with day-night temperatures of 23 and 19°C, respectively. From the first week on, 8mL of modified Hoagland solution were applied weekly till harvest time. Pots were randomly distributed in the growth chamber under 355 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity. Unshaded plants were grown under normal light till harvest time. For shaded treatments, shade cloth was stretched over wire frames (60 cm in height, 50% shade) and placed over individual pots before the end of the night. After 24, 48 and 72 hours (corresponding to 16, 32 and 48 hours of shading stress) three plants from each treatment were removed and harvested. Roots and shoots were harvested and immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Transcript accumulation in roots was assessed by relative changes, expressed as response ratios, *i.e.* x-fold factors compared to a control condition

3. Results

3.1. Phylogenic and sequence analysis of MST5 and MST6

Two full-length sequences (*RiMST5* and *RiMST6*) belonging to the major facilitator superfamily (MFS) were identified, both presenting high similarity to genes encoding fungal MSTs (*e.g.*: *Geosiphon pyriformis* *MST1* and *Botryotinia fuckeliana* *FRT1*). Phylogenetic analysis of several fungal monosaccharide transporters (Fig. 1) showed that *RiMST5* and *RiMST6* cluster with fungal glucose transporters, including *RiMST4* and *GpMST1* (Helber et al., 2011; Schüßler et al., 2006, 2007); while *RiMST2* and *RiMST3* cluster with xylose transporters. Interestingly, yeast and ectomycorrhizal hexose transporters formed two separated clusters distant from endomycorrhizal clusters. This segregation supports the analysis of Schüßler et al. (2006) that demonstrated the presence of large phylogenetic distance between Glomeromycota and the Ascomycota /Basidiomycota lineage; and highlights this phylogenetic distance between ecto- and endomycorrhizal MST sequences. Further analyses of the deduced proteins showed that *RiMST5* and 6 both possess 12 transmembrane domains and the conserved cd06174 motif, confirming the characteristic features of the MFS. *RiMST5*, but not *RiMST6*, contained the PRK10077 motif (xylE motif of d-Xyl transporters, E-value 2.90×10^{-47}). On the contrary *MST6*, and not *MST5*, contained the highly conserved pentameric amino acid motif R-X-G-R-K, an important determinant of membrane topology of the MFS members. Thus, both *RiMST5* and 6 contain strong signature motifs previously identified in sugar transporters.

3.2. *RiMSTs* biochemical characterization

The open reading frames of *RiMST5* and *RiMST6* were cloned into the yeast expression vector pDRf1-GW and transferred to the mutant yeast strain EBY.VW4000 impaired in monosaccharide uptake. Functional complementation was clearly obtained for *RiMST6*-expressing strains when grown on glucose as the sole carbon source (Fig. 2). Also, the functional complementation was improved on higher glucose concentrations (22, 50 and 150mM) even if the growth was weaker than for *GpMST1*. For *RiMST5*-expressing strains, a weaker complementation was observed on SD ura⁻ plates (Fig. 2). To check the possible effect

of the pH, which might be partly responsible of the yeast growth variations, we performed drop test complementation assays at various pH levels. Using this approach, we obtained improved growth of *RiMST5*-expressing strains at pH 6 when compared to the empty vector control; this transporter showed a weaker complementation than *RiMST6*-expressing strains (Fig. S1A).

Furthermore, the functionality of these transporters was verified by measuring optical density variation (OD_{600}) of *RiMST5* and *RiMST6* expressing strains growing on the selective synthetic medium (SD ura-) supplemented with 150 mM glucose (Fig. 3A). The two mutant strains grew on this selective medium with different efficiencies, thus confirming their ability to uptake and use glucose and their functionality as glucose transporters. The mutant harboring pDRf1-*RiMST6* showed a typical yeast growth curve with log and stationary phases, while a weaker growth rate was observed for the yeast transformed with pDRf1-*RiMST5* (Fig. 3A). The expression analysis of *MST5* transformed yeast confirmed that the low growth is related to the presence of the transporter (Fig. S1B).

To confirm the monosaccharide transport activity of *RiMST5* and *RiMST6*, we performed [^{14}C]-glucose uptake assays. By this mean, we characterized *RiMST5* as a high affinity functional transporter with a K_m of 140 μM and a V_{max} of 13.8 nmol of glucose min^{-1} (10^8 cells) $^{-1}$ at pH 5.5 (Fig. 3B). Compared to *RiMST5*, *RiMST6* also exhibits a high affinity for glucose, albeit lower than *RiMST5*, with an estimated K_m of 0.8 mM (Fig. 3C, Fig. S2), and a V_{max} of 23.1 nmol of glucose min^{-1} (10^8 cells) $^{-1}$ at pH 5.5. In addition, investigation of transport dependency on membrane potential revealed significantly reduced or suppressed transport properties of *RiMST6* upon addition of protonophores or H^+ -ATPase inhibitors (Fig. 4A), revealing that *RiMST6* is an active H^+ /glucose co-transporter. During substrate competition assays, *RiMST6* showed high specificity to glucose (Fig. 4B), with no affinity for other substrates such as mannose, xylose, galactose, fructose and sucrose. These assays could

not be successfully performed for RiMST5 in regard to the lower capacity measured in the labelled glucose uptake studies.

3.3. *RiMSTs* expression pattern and host-plant effect

Transcript accumulation of the newly characterized *RiMST5* and *RiMST6*, as well as *RiMST2*, *RiMST3* and *RiMST4* was assessed by quantitative-real time-PCR (q-RT-PCR) in (i) 10-day old germinating spores, a development stage of AMF before root colonization, and (ii) during symbiosis with *M. truncatula* roots. As already demonstrated, *RiMST2* was highly expressed during the *in planta* phase and its transcript levels were ten times lower in germinated spores. *RiMST6* was expressed at similar levels in the two AMF developmental stages, whereas *RiMST5* transcripts accumulated 10-fold more in germinated spores than in mycorrhizal roots (Fig. 5). Furthermore, transcript accumulation of all *RiMSTs* was assessed in *S. bicolor* (C₄ plant), *P. trichocarpa* (C₃ plant) and *M. truncatula* (C₃ plant) roots during the interaction with *R. irregularis* DAOM 197198, as well as in the ERM (Fig. 6). Although *RiMSTs* transcripts varied according to plant species, no evident correlation with the carbon dioxide fixation pathways (C₃ or C₄) was noticed.

3.4. *RiMSTs* expression under light stress conditions

RiMSTs transcript profile was assessed in mycorrhizal plants under partial deprivation of light for different periods in order to understand how sugars uptake by the fungus is affected in carbon-stressed plants. Taking into account that *S. bicolor* invest heavily in carbon transfer to the fungal partner during mycorrhizal interactions (Walder et al., 2012), it was relevant to use it as a host plant for a shading experiment to better visualize the regulation of *MST* expression under C-deprivation. We grew *S. bicolor* mycorrhizal plants for three months and then shaded the plants for 24, 48 or 72 hours. The expression of all *MST* genes (*RiMST2-6*) was stable in shaded plants or somewhat lower than in unshaded plants after 24 hours, but RiMST2, RiMST4 and RiMST5 were transiently up-regulated after 48 hours (Fig. 7).

4. DISCUSSION

4.1. MST5 and MST6, new members of the *R. irregularis* MST family

In the present work, we reported the identification of two new monosaccharide transporters (MST) from *R. irregularis*, bringing the current total to 5 identified MSTs in *R. irregularis*. RiMST5 and RiMST6 emerged as functional transporters with apparent K_m for glucose of 0.14 mM and 0.8 mM, respectively. The previously characterized glucose transporter RiMST2 was described as a “high-affinity transporter” for glucose with a K_m of 33-12.5 μ M (SE) that can transport a wide range of substrates (Helber et al., 2011). Compared to this versatile MST, RiMST6 appeared to be a high-affinity transporter that would be an exclusive glucose transporter, unless another not yet tested substrate is shown to be transported and that therefore might have a major role in glucose uptake. The K_m of both RiMST5 and RiMST6 (compared to other MSTs, Table S1) suggested that they allow *R. irregularis* to rapidly take up sugar molecules into the cells during starvation, representing a valuable mechanism during microbial competition for sugar access in the soil. The same idea was previously suggested by Delgado-Jarana et al. (2003) who showed that *gtt1*, a high-affinity glucose transporter ($K_m=12 \mu$ M) from the ascomycete *Trichoderma harzianum* is strongly induced in conditions of glucose starvation or when this mycoparasite is confronted with the fungus *Rhizoctonia solani*. Taking this into account, RiMST5 and RiMST6 could also be involved during root colonization in conditions of (i) competition with other rhizospheric microbes (e.g. pathogens); (ii) low sugar transfer to the root compartment (e.g. photosynthesis efficiency reduction in regard to new light conditions (competition between plants or to grazing for example) or (iii) direct sugar uptake from the soil which contains low concentrations of glucose.

In the context of AMF, due to the fungal obligate biotrophy, germinating spores must develop rapidly to reach the host plant and initiate a symbiotic interaction ensuring substantial carbon supply. Here, based on the high expression of *RiMST5* in germinated spores, we

postulated that RiMST5 may also participate to glucose uptake from the soil, mainly contributing to sustaining a “vital” sugar concentration in fungal tissues, especially during spore germination, which is a critical step in the fungal life cycle, but also during hyphal development before efficiently interacting with a host cell. Finally, when compared to *RiMST2*, which is mostly expressed during *in planta* phase, RiMST5 and RiMST6 seem to play a different and most probably complementary role in fungal sugar uptake.

Carbon uptake during mycorrhizal interactions is made possible thanks to a versatile efficient monosaccharide transport system. In *Saccharomyces cerevisiae*, more than 20 genes encode monosaccharide transporters and their expression is controlled by glucose sensors according to the availability of the carbon sources (Boles and Hollenberg, 1997). Furthermore, screenings for putative hexose transporter genes revealed a similar multigenic system in the genome of pathogenic fungi as *Neurospora crassa* (Galagan et al., 2003). More recently, in the ectomycorrhizal fungus *Laccaria bicolor* (López et al., 2008), 15 putative hexose transporters were identified and classified into two groups: those involved in carbon uptake by soil-growing hyphae and those involved in hexose transfer at the plant-fungus interface. Accordingly, the presence of a multigenic hexose transport system regulated by development and carbon nutrition during AM symbiosis is strongly expected. The next and foremost step for deciphering sugar partitioning in AM is the identification and characterization of all fungal sugar transporters.

4.2. MST expression of *R. irregularis* is host plant dependent

Most studies on carbon-partitioning in mycorrhizal plants do not emphasize the importance of the host plant species involved in AM symbiosis, while various studies highlight the effect of different fungal species on the symbiotic interaction (Lerat et al., 2003; Kiers et al., 2011). Carbon-sink strength and carbon-partitioning in AM was showed to depend on both symbiotic partners in a split-root system (Lerat et al., 2003). Moreover, using compartmented

microcosms with mono and mixed cultures, Walder et al. (2012) tracked the carbon source of AMF and discovered marked asymmetry in terms of trade: flax (C3 plant) invested little carbon but gained up to 94% of the nitrogen and phosphorus provided by the fungus, whereas neighboring *S. bicolor* (C4) invested massive amounts of carbon with little return. These results prove the importance of the host plant in the carbon-partitioning of mycorrhizae.

Among all transporters, *RiMST2* was the only one that kept a high expression level in mycorrhizal roots compared to the ERM in all the inoculated plants, supporting the fact that *RiMST2* is the major component for hexose uptake by *R. irregularis*. In fact, Helber et al. (2011) showed that HIGS-mediated RNAi silencing of *RiMST2* resulted in impaired mycorrhizal interaction, defective arbuscules and reduced *MtPT4* expression. *RiMST2* is therefore indispensable for a functional AM symbiosis.

We also noticed higher expression of *RiMST2* in *S. bicolor* mycorrhizal roots; which could be explained by the high quantity of carbon invested by the plant, as shown by Walder et al., 2012. Our results also suggested that the localization of transcript accumulation of *RiMST3*, *RiMST4*, *RiMST5* and *RiMST6* (unlike *RiMST2*) depends on the host plant and probably on monosaccharide availability. In mycorrhizal *S. bicolor* roots, *RiMST2* and *RiMST4* are induced by the high concentration of monosaccharide supply, while *RiMST3*, *RiMST5* and *RiMST6* are repressed. Such a regulation by available glucose concentrations has already been described for *S. cerevisiae* Hxts (Reifenberger et al., 1997). Moreover, our results showed that the lower expression of *RiMST5* and *RiMST6* in *S. bicolor* roots is correlated with a higher expression in the ERM, supporting our hypothesis that the amount of sugar delivered by the plant regulates MST expression and localization. Similar complementary expression profiles for *RiMST5* and *RiMST6* were observed for *P. trichocarpa*. This also suggested a regulation of the expression of both monosaccharide transporters by available glucose. Our results unveil a complex role of

fungal RiMSTs, which appear to be dependent on both the host plant and the available sugar levels during AM symbiosis.

4.3. Fungal sugar uptake is affected by light availability

Plants adjust their capacity to carry out photosynthesis in response to the demand for photosynthetic products. This balance between source (photosynthetates) and sinks is continuously adjusted in response to environmental and developmental cues (Paul and Foyer, 2001). Interestingly, when photosynthetic carbon fixation is reduced, photosynthesis is not primarily affected. Phloem transport of sugars is the first to be affected, leading to an increased sucrose concentration in leaves and a feed-back inhibited photosynthesis (Ainsworth and Bush, 2011). Little is known about the regulation of plant and fungal sugar transporters during light stress at the molecular level but many studies have been conducted at the physiological level. Namely, after 24 hours of shade, photosynthesis is generally light-limited and sink demand is expected to exceed carbon assimilation. However, plants with apoplastic sugar loading offer greater flexibility for photosynthetic acclimation upon transfer from one light environment to another (Amiard et al., 2005). In addition, after a short stress, regulation of starch synthesis and degradation may participate to the coordination of carbon supply and carbon utilization (Stitt et al., 2010). Thus, we do not expect any feedback on sugar uptake from the fungus at this stage.

The up-regulation of almost all *RiMSTs* after 48 hours of shading points out to a drop in photosynthetic C supply, *i.e* a lower concentration of monosaccharides at the symbiotic interface. This up-regulation also reflects a higher demand for monosaccharides by the fungus and supports the hypothesis of its capacity to gain control, at least transiently, over the symbiotic trade despite its high host dependency (Fellbaum et al., 2012). After 72 hours of shading, the expression of *RiMSTs* returned to normal levels (the expression of *MSTs* in unshaded roots was comparable to the expression in shaded plants). In this experiment, shading the plant host had no significant effect on *MST6* expression, which is possibly due to the short

duration of the stress and the presence of sufficient glucose concentration in the symbiotic interface (unable to induce *MST6* up-expression). This hypothesis is supported by preliminary results on *S. bicolor* that have undergone 48 hours of darkness, showing an up-regulation of *MST6* (Fig. S3). This tuned-up expression reveals a critical role of *MST2*, *MST4* and *MST5* in glucose uptake from the symbiotic interface and their importance for the outcome of the AM symbiotic interaction.

Our results suggest that under carbon-limiting conditions, the “fair trade” principle is set aside and a relationship based on “first come-first served” takes over. Indeed, increasing the plant carbon cost by shading does not reduce the carbon flow to the fungus and nor decreases colonization levels in *Trifolium subterraneum* colonized by *R. irregularis* (Olsson et al., 2010). This indicates that *R. irregularis* may act as a parasite under certain conditions. Johnson et al. (1997) suggested that fertilization (reduced benefits) or shading (increased costs) could generate mycorrhizal parasitism. More recently, Bahn et al. (2013) showed that sustained shading depleted the carbohydrate pool in the shoots but not in the roots and that fungal carbon-sink strength was not affected by the reduced carbon source strength.

Further molecular evidence of a competition for apoplastic glucose between plant and fungus has been provided by several studies. Some plant *MSTs* are involved in sugar resorption upon infection by pathogens (Slewinski, 2011). This was supported by the correlation between the up-regulation of the plant cell-wall invertase *AtβFRUCT1* and the hexose transporter *AtSTP4* in *Erysiphe cichoracearum*-infected *Arabidopsis* leaves (Fotopoulos, 2003). Additional evidence was provided in infected broad bean by the characterization of rust *Uromyces fabae* sugar transporter *UfHXT1* localized in the haustoria. *UfHXT1* preferentially transports glucose and fructose towards the fungus (Voegelé et al., 2001). Substrate specificity and localization of such fungal *MSTs* facilitate hexose uptake from the host and thus participate in fungal sink strength. Lemonnier et al. (2014) recently showed a connection between glucose transporter

AtSTP13 transcripts, protein accumulation, glucose uptake rates and the resistance level to *Botrytis cinerea* infection. The contribution of *AtSTP13* to basal resistance to *Botrytis cinerea* highlighted an active reuptake of apoplastic hexoses possibly involved in starvation of the fungus.

In this paper, we report about the identification and roles of two new monosaccharide transporters from *R. irregularis* (RiMST5 and RiMST6). We demonstrate for the first time, that both fungal monosaccharide expression level and localization vary with the host plant, thereby suggesting a versatile role for MSTs, as dependent upon plant investment in the symbiotic interaction. We also show that *MSTs* expression levels respond to the plant lighting intensity (shade/darkness), indicating that MSTs may play a role in enhancing the fungal sugar strength during starvation. This work highlights an additional complexity in the functioning of mycorrhizal associations and opens new perspectives onto our knowledge about carbon partitioning during plant-microbe interactions.

Author contributions

The work presented here was carried out in collaboration between all authors. Ait Lahmidi N., Doidy J., Courty P-E., Wipf D. and Bonneau L. designed the experiments and interpreted the results. Ait Lahmidi N., Brulé D. , Arnoud C. and Chatagnier O. carried out the experiments and analyzed the data. Ait Lahmidi N. carried out phylogenetic analysis and drafted the article. Berta G., Lingua G., Wipf D., Courty P-E. and Bonneau L. revised the article.

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Figure captions

Figure 1: Phylogenetic analyses of a multiple alignment of the deduced protein sequences of *R. irregularis* MST5, MST6 and other fungal monosaccharide transporters. MSTs from the following fungi were included: *Amanita muscaria*; *Arthroderma Otae*; *Aspergillus fumigatus*; *Aspergillus kawachii*; *Aspergillus niger*; *Aspergillus oryzae*; *Blumeria graminis*; *Botrytis cinerea*; *Botryotinia fuckeliana*; *Debaryomyces hansenii*; *Geosiphon pyriformis*; *Laccaria bicolor*; *Rhizophagus irregularis* (labeled with a black asterisk) ; *Tuber borchii*; *Saccharomyces cerevisiae*; *Scheffersomyces stipites*; *Sclerotinia borealis*; *Sclerotinia sclerotiorum*; *Uromyces viciae-fabae*. The sequences of the sugar transporters ST1 from *M. truncatula* (MtST1) and *Zea mays* (ZmST1) were used as outgroups. The clade of yeast monosaccharide transporters (green) and the clade of ectomycorrhizal hexose transporters (blue) are found distant from the Glomeromycota hexose transporter sequences. MST2 and MST3 cluster together with known fungal xylose transporters while MST4, MST5 and MST6 cluster close to the GpMST1. The dendrogram was generated using Mega 4.0 and Muscle software for the alignment and the Maximum parsimony method to construct the phylogeny (Tamura *et al.*, 2007). Heuristic tree searches were performed using 1,000 random sequence additions and the Subtree-Pruning-

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Figure 2: Complementation of a yeast mutant defective in glucose uptake by RiMST5 and RiMST6.

Growth of the yeast strain EBY.VW4000, transformed with various constructs, on selective synthetic medium supplemented with various glucose concentrations (SD ura- with 22, 50 or 150 mM glucose) as the sole carbon source and on YPD medium. All strains were incubated for 6 days at 30°C. **(1)** GpMST1, $\Delta hxt1-17$ + pDRf1-GpMST1; **(2)** RiMST6, $\Delta hxt1-17$ + pDRf1-RiMST6; **(3)** RiMST5, $\Delta hxt1-17$ + pDRf1-RiMST5 and **(4)** Empty vector (control) $\Delta hxt1-17$ + pDRf1.

Figure 3: Biochemical characterization of RiMST5 and RiMST6

(A) Growth curves (optical density vs. growth time) of *S. cerevisiae* yeast strains EBY.VW4000 expressing *RiMST5* or *RiMST6* and grown on selective SD ura- medium (150 mM glucose) at 30°C for 7 days with data measurements every hour. Yeast transformed with the empty vector pDRf1-GW was used as a control. At least 6 biological repetitions per candidate have been performed for the growth assay. **(B)** Time-course of [14 C]-glucose uptake by the yeast strain EBY.VW4000 expressing *RiMST5* at the indicated sucrose concentrations at pH 5.5. The Michaelis-Menten kinetics of glucose uptake rates yielded a K_m of 0.14 mM. **(C)** Time-course of [14 C]-glucose uptake by the yeast strain EBY.VW4000 expressing *RiMST6* at the indicated sucrose concentrations at pH 5.5. Michaelis-Menten kinetics of glucose uptake rates yielded a K_m of 0.8 mM.

Figure 4: (A) Influence of plasma membrane energization on the uptake rate of [^{14}C]-glucose in the yeast mutant EBY.VW4000 expressing *RiMST6*. Incubation in the presence of protonophores (CCCP, DNP) and plasma membrane H^+ -ATPase inhibitors (DES, Vanadate) for 5 min. (B) Substrate specificity of *RiMST6*. (A-B) Data are expressed as percentages of the glucose control and values represent the means of at least three independent experiments. Differences in relative uptake between the treatments were performed with a Kruskal-Wallis test. Stars (*) indicate a P -value < 0.05 .

Figure 5: Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *RiMST2*, *RiMST3*, *RiMST4*, *RiMST5* and *RiMST6* in colonized *M. truncatula* roots and germinated *R. irregularis* spores. The values are the means of three biological replicates, and error bars represent SE. Transcription Elongation Factor (TEF) was used as the reference transcript. Differences in gene expression between the treatments were performed with a Kruskal-Wallis test. Letters indicate a P -value < 0.05 and **nd** correspond to non-detected expression.

Figure 6: Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *RiMST2*, *RiMST3*, *RiMST4*, *RiMST5* and *RiMST6* in *M. truncatula*, *S. bicolor* and *P. trichocarpa* mycorrhizal roots and in the corresponding extra-radicular mycelium (ERM). The values are the means of three biological replicates, and error bars represent SE. Transcription Elongation Factor (TEF) was used as the reference transcript. Differences in gene expression between the treatments were performed with a one-way ANOVA (Scheffe's F-test). Letters indicate a P -value < 0.05 . Independent statistical analyses

were performed for each gene, with capital letters for mycorrhizal root tissues and lowercase for ERM.

Figure 7: : Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *RiMST2*, *RiMST3*, *RiMST4*, *RiMST5* and *RiMST6* in *S. bicolor* mycorrhizal roots after 24, 48 and 72 hours of shade (S) compared to unshaded plants (L). The values are the means of three biological replicates, and error bars represent SE. Transcription Elongation Factor (TEF) was used as the reference transcript. Differences in gene expression between the treatments were performed with a one-way ANOVA (Scheffe's F-test). Letters indicate a *P*-value < 0.05.

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A

B

C

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Figure 5: Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *RiMST2*, *RiMST3*, *RiMST4*, *RiMST5* and *RiMST6* in colonized *M. truncatula* roots and germinated *R. irregularis* spores. The values are the means of three biological replicates, and error bars represent SE. Transcription Elongation Factor (TEF) was used as the reference transcript. Differences in gene expression between the treatments were performed with a Kruskal-Wallis test. Letters indicate a P -value < 0.05 and **nd** correspond to non-detected expression.

Figure 6: Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *RiMST2*, *RiMST3*, *RiMST4*, *RiMST5* and *RiMST6* in *M.*

truncatula, *S. bicolor* and *P. trichocarpa* mycorrhizal roots and in the corresponding extra-radicular mycelium (ERM). The values are the means of three biological replicates, and error bars represent SE. Transcription Elongation Factor (TEF) was used as the reference transcript. Differences in gene expression between the treatments were performed with a one-way ANOVA (Scheffe's F-test). Letters indicate a P -value < 0.05 . Independent statistical analyses were performed for each gene, with capital letters for mycorrhizal root tissues and lowercase for ERM.

Figure 7: Quantification by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of the transcript abundances of *RiMST2*, *RiMST3*, *RiMST4*, *RiMST5* and *RiMST6* in *S. bicolor* mycorrhizal roots after 24, 48 and 72 hours of shade (S) compared to unshaded plants (L). The values are the means of three biological replicates, and error bars represent SE. Transcription Elongation Factor (TEF) was used as the reference transcript. Differences in gene expression between the treatments were performed with a one-way ANOVA (Scheffe's F-test). Letters indicate a P -value < 0.05 .