Knockdown of the Symbiotic Sucrose Synthase MtSucS1 Affects Arbuscule Maturation and Maintenance in Mycorrhizal Roots of Medicago truncatula

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The relevance of the symbiosis-induced Medicago truncatula sucrose synthase gene MtSucS1 for an efficient arbuscular mycorrhiza (AM) was studied using two independent antisense lines that displayed up to 10-fold reduced SucS1 levels in roots. Mycorrhizal MtSucS1-reduced lines exhibited an overall stunted aboveground growth under inorganic phosphorus limitation. Apart from a reduced plant height, shoot weight, and leaf development, a delayed flowering, resulting in a lower seed yield, was observed. In addition, the root-to-shoot and root weight ratios increased significantly. Gene expression studies demonstrated a major reversion of AM-associated transcription, exhibiting a significant repression of well-known plant AM marker and mycosymbiont genes, together indicating a diminished AM fungus colonization of MtSucS1-antisense lines. Concomitantly, gas chromatography-mass spectrometry-based metabolite profiling revealed that mycorrhizal MtSucS1-reduced lines were affected in important nodes of the carbon, nitrogen, and phosphorus metabolism, accentuating a physiological significance of MtSucS1 for AM. In fact, antisensing MtSucS1 provoked an impaired fungal colonization within the less abundant infected regions, evident from strongly reduced frequencies of internal hyphae, vesicles, and arbuscules. Moreover, arbuscules were early senescing, accompanied with a reduced development of mature arbuscules. This defective mycorrhiza status correlated with reduced phosphorus and nitrogen levels and was proportional to the extent of MtSucS1 knockdown. Together, our results point to an important role for MtSucS1 in the establishment and maintenance of arbuscules in the AM symbiosis.

The arbuscular mycorrhiza (AM) with endotrophic fungi (AMF; Smith and Read, 1997) of the phylum Glomeromycota (Schüßler et al., 2001) is the most important mutual symbiosis of land plants (Smith and Read, 1997). The beneficial nutrient exchange of carbon (C) for mainly phosphorus (P), nitrogen (N), and enhanced water availability at the plant/fungus interface is central to AM (Smith and Smith, 1990, 1997). Moreover, AM interactions support plant growth and reproduction (Valentine et al., 2001; Wu and Xia, 2006) as well as the host’s resistance and tolerance to pathogens and abiotic stress conditions (Liu et al., 2007; van Wees et al., 2008). For up to 70% of the host plant P (Smith and Read, 1997), obligate biotrophic AMF receive their entire C requirement from an approximately 20% to 30% proportion of recently assimilated C (Jakobsen and Rosendahl, 1990; Douds et al., 2000; Bago et al., 2003).

In plants, a substantial portion of the photosynthetically fixed C is channeled into Suc synthesis. Suc is used for long-distance C and energy transport into diverse heterotrophic sinks and represents the preferred carbohydrate (CH) translocated to AM, which is associated with an efficient C flux into mycosymbionts (Bücking and Shachar-Hill, 2005). The symbiotic sink diverts the Suc flow to AM-colonized sites (Blee and Anderson, 2002; Heinemeyer et al., 2006; García-Rodríguez et al., 2007), causing considerably lower total free CH contents in mycorrhizae than in nonmycorrhizal (NM) roots. Photosynthetic limitations were reported to affect mycorrhizal growth responses and inorganic P (Pi) uptake (Tester et al., 1985). Here, the CH shortage in symbiotic roots lowered or inhibited the AMF colonization (Son and Smith, 1988; Vierheilig et al., 2002) and decreased the proportion of functional arbuscules (Hayman, 1974). Consequently, photosynthetic activity, CH metabolism, and transport processes are optimized for symbiosis maintenance (Wright et al., 1998a, 1998b; Bláck et al., 2000), providing substrates for the higher respiratory demand of mycorrhizae (Shachar-Hill et al., 1995; Douds et al., 1999).
2000). This optimization is supported by altered expression profiles of genes involved in Suc breakdown (Blee and Anderson, 2002; Hohnjec et al., 2003; García-Rodriguez et al., 2007) and sugar transport (Harrison, 1996; García-Rodriguez et al., 2005).

So far, details on the AM-associated CH translocation at the symbiotic interface are limited (Pfeffer et al., 1999; Bago et al., 2003). It is still a matter of debate whether plants actively regulate this process (Graham et al., 1997) and whether intraradical hyphae (Solaïman and Saito, 1997; Bago et al., 2003) or arbuscules are the principal sites of fungal CH uptake (Pfeffer et al., 1999; Lammers et al., 2001). Due to the facts that intraradical fungal structures are unable to take up and utilize Suc (Shachar-Hill et al., 1995; Solaïman and Saito, 1997) and that Suc-cleaving activities are not identified in AM yet (Schubert et al., 2003), exogenous hexoses (mainly Glc) have to be supplied to the myc symbionts (Shachar-Hill et al., 1995; Bücker and Schachar-Hill, 2005). Interestingly, Schüßler et al. (2006) characterized the first glomeromycotan H+-monosaccharide cotransporter (GpMST1), presumably operating in the fungal arbuscular membrane of the mutual Geosiphon pyriformis/Nostoc punctiforme system.

Prior to the utilization by AMF, Suc must thus be hydrolyzed by the plant either via cytoplasmic, cytoplasmic Suc synthases (SucS; Huber and Akazawa, 1986) and/or by different apoplastic and cytoplasmic invertases (Inv; Stur, 1999), particularly in the arbuscule-containing cells. In general, enhanced total Suc-cleaving activity involving different Inv and SucS followed the colonization of clover (Trifolium repens) roots with a mixed population of AMF (Wright et al., 1998a). Regarding the importance of extracellular, acidic Inv during AM, Schaarschmidt et al. (2007a) showed that, along with a root-specific inhibition of cell wall (CW)-bound, apoplastic Inv activity in transgenic tobacco (Nicotiana tabacum), the AM status was reduced. Additionally, transcriptional induction of the extracellular Inv gene LIN6 was located near AM structures and in phloem cells of mycorrhizal tomato (Solanum lycopersicum) roots (Schaarschmidt et al., 2006). However, artificially augmented hexose availability, obtained by yeast-derived, apoplastic Inv active in the arbuscule interface of transgenic mycorrhizal Medicago truncatula roots, failed to improve AM significantly (Schaarschmidt et al., 2007a; Schaarschmidt and Hause, 2008).

With respect to the involvement of cytoplasmic SucS during AM, transcript accumulation of two SucS genes was differentially affected in AM-colonized maize (Zea mays) roots (Ravn skov et al., 2003). For the Glycine max/Glomus mosseae interaction, Schubert and coworkers (2003) showed that, along with a cytosolic alkaline Inv, also SucS activities significantly increased, while both soluble acid and apoplastic, CW-bound Inv activities diminished during AM development. On the other hand, Blee and Anderson (1998, 2002) ascertained that SucS-mediated and soluble, potentially vacuolar acid Inv-mediated C availability affected the regulation of arbuscule location and function in Phaseolus vulgaris.

Given these somewhat controversial findings regarding AM-associated Suc breakdown, we attempted to study the relevance of an efficient, SucS-mediated symplastic sink near the plant/fungus interfaces. In M. truncatula, the SucS family consists of five differentially regulated genes (Hohnjec et al., 1999, 2003). Of these, the MtSucS1 gene was most strongly expressed in root tissues, and only MtSucS1 was activated under endosymbiotic conditions. This was demonstrated by real-time reverse transcription (RT)-PCR (Hohnjec et al., 2003) and is supported by in silico expression analyses of the corresponding tentative consensus sequence TC100410 (M. truncatula Dana-Farber Cancer Institute Gene Index; http://compbio.dfci.harvard.edu/). In reporter gene assays of mycorrhizal roots, the MtSucS1 promoter was specifically activated in arbuscule-containing cells and cortical cells of the surrounding root regions (Hohnjec et al., 2003). Together, the characteristic MtSucS1 inductions in AM and root nodules suggest a physiological importance of MtSucS1 orthologues in legume/microbe interactions in general (Hohnjec et al., 2003; Baier et al., 2007).

Having shown the importance of MtSucS1 for biological N fixation in root nodules (Baier et al., 2007), we here employed the same MtSucS1-antisense (MtSucS1-as) lines to assess the function of MtSucS1 under AM conditions and investigated physiological effects occurring downstream of the reduced SucS1-mediated sink strength. Integrating physiological analyses and molecular techniques, we report a reduced growth and reproduction, photosynthetic activity, and an impaired nutritional status when MtSucS1-as lines depended on an efficient AM symbiosis. These physiological limitations were accompanied by repressions of plant and fungal AM marker genes and altered metabolite responses upon MtSucS1 reduction in endosymbiotic root tissues. Moreover, antisensing MtSucS1 in the two independent lines coincided with a defective internal AMF colonization. Apart from the reduced total root length colonization, deficiencies of the fungal occupancy of the roots were particularly evident from significantly decreased numbers of arbuscules within colonized regions and, strikingly, their handicapped development and earlier senescence. In line with our recent finding that MtSucS1 reduction affects symbiotic N fixation in M. truncatula root nodules (Baier et al., 2007), our results extend the current understanding of symbiotic legume/microsymbiont interactions toward plant/fungus nutrient exchange in AM.

RESULTS

To study the relevance of the symbiosis-induced Suc synthase MtSucS1 for the M. truncatula/G. mosseae mycorrhiza, two independent, transgenic MtSucS1-as lines (MtSucS1-as12 and -as19) were used, exhibiting approximately 10% and 20% of wild-type MtSucS1
transcript and protein levels in roots and root nodules, respectively (Baier et al., 2007). Phenotypic patterns of mycorrhizal, P$_i$-limited MtSucS1-as lines were compared with two mycorrhizal transgenic control lines. These independent transformation controls (subsequently named c) expressed fusions of the MtSucS1 promoter to the intron-containing gusA reporter gene (gusA), maintained MtSucS1 wild-type levels in roots and root nodules (Baier et al., 2007), and exhibited phenotypic patterns comparable to equally treated roots and root nodules (Baier et al., 2007) and exhibited equal photosynthetic performances and pod yields under nonsymbiotic growth conditions (Supplemental Table S1, A and B; Supplemental Fig. S1, A and B).

At final harvest, the total fresh weights of the mycorrhizal lines hardly varied, but 10% and 21% reduced dry weight-to-fresh weight ratios indicated altered water relations and/or cell densities in mycorrhizal MtSucS1-as12 and -as19, respectively. Deducing from the positive mycorrhizal growth responses (MGRs; growth responses to Glomus colonization as increase in aboveground dry weight; Fig. 2B), all lines benefited from mycorrhization. Nevertheless, MGRs of the antisense lines were depleted considerably, with the MGR of line as19 being reduced by 27% and that of line as12 by 49% compared with control levels. Accordingly, mycorrhizal MtSucS1-as12 developed an average shoot dry weight of 75% and as19 of 82% (Fig. 2B), reflecting the temporal aboveground development (Figs. 1 and 2, A and B). While the root fresh weights of MtSucS1-as lines were not significantly altered, the relative root growth increased in mycorrhizal MtSucS1-as lines. In fact, the root-to-shoot ratios (RSR) resulted in a ranking of as19 > as12 > c in the ratio 1.51:1.25:1 (Fig. 2B). In contrast, for fully supplemented MtSucS1-as12 and -as19 lines, the RSR (0.57 and 0.80, respectively) ranged near the full supplementation controls (0.60; Supplemental Table S1A) as well as near the RSR of 0.75 to 0.80 common for plants grown under nonstressed conditions (Waring et al., 1998). Compared with transformation controls, the fully supplemented line as19 already showed a tendency for an enhanced belowground allocation (Supplemental Table S1, A and D), but alterations in the biomass partitioning pattern were solely significant when depending on an efficient AM symbiosis (Supplemental Fig. S1D).

Alterations in the degree of root system plasticity are mirrored in variables of the root system architecture (RSA; Fig. 2C). Here, the total root length (TRL), root surface area (RSA), root volume (RV), and root tip numbers (RT) are sufficient parameters to describe root functions such as nutrient and water acquisition. In response to P$_i$ limitation, according to their TRL, the ranking of AM lines was as19 > as12 > c in the ratio 1.90:1.14:1 (Fig. 2C). Hence, MtSucS1-as19 established the largest root system and also mirrored average 1.8-fold increases on RV, RSA, and RT, depending on the substantially lower levels throughout the experimental period (Fig. 2A). Moreover, particularly line as12 exhibited an approximately 10-d earlier senescence of older leaves. During an active AM stage (28 dpi), the C-fixing performance, measured as the photochemical quantum yield of the youngest and oldest source leaf pair, tended to decline with leaf age independently of the genetic background but biased significantly stronger mycorrhizal MtSucS1-as lines (Supplemental Fig. S2). Whereas the initiation of inflorescences commenced about 19 dpi in AM control lines, this onset was delayed by 5 d in mycorrhizal MtSucS1-as12 and by 9 d in MtSucS1-as19, resulting in considerably reduced yield at 50 dpi (Fig. 2B). Notably, MtSucS1-as and control lines exhibited equal photosynthetic performances and pod yields under nonsymbiotic growth conditions (Supplemental Table S1, A and B; Supplemental Fig. S1, A and B).

MtSucS1 Reduction Affects Plant Growth under AM-Dependent, P$_i$-Limited Conditions

All phenotypic AM analyses were conducted on glasshouse-grown, G. mosseae-colonized plants cultured under strict P$_i$-deprived conditions from the day of inoculation until their final harvest at 50 d post inoculation (dpi). Depending on the symbiotic P supply, mycorrhizal MtSucS1-reduced plants exhibited an overall stunted vegetative growth in terms of plant height and leaf development. Along with the stronger MtSucS1 knockdown in line as12, these aboveground alterations were more pronounced here (Fig. 1). Calculated over the experimental period, phyllotaxic rates such as the initiation of sequential leaf primordia (rate of leaf initiation) and the successive development of two fully expanded leaves (rate of leaf appearance) were significantly reduced in both MtSucS1-as lines (Supplemental Table S2). This led to significantly elongated plastochrons and phyllochrons of the mycorrhizal antisense lines that therefore needed more time to initiate and completely develop two successive leaves, respectively (Supplemental Table S2). Classifying four developmental stages of leaf organogenesis from leaf primordia to a fully expanded source leaf by a decimal code (Baier et al., 2007), at 14 dpi, leaf rate curves of both MtSucS1-as lines started to differentiate significantly from controls and leaf units remained at
symbiotic P\textsubscript{i} acquisition (Fig. 2C). The TRL of the mycorrhizal line as12 was increased by 14%, and other absolute RSA parameters ranged between minor differences to a 24% enhancement of RTs, albeit changes were not significant. In both MtSucS1-as lines, the elevated root system growth coincided with minor but significant reductions of the average root diameter (~4% and ~6% in MtSucS1-as12 and -as19 mycorrhiza, respectively). Along with altered biomass partitioning patterns, these differences in the RSA were even more dramatic when calculated with respect to the production of shoot dry weight mediated by the root system produced. Thus, mycorrhizal antisense lines differed markedly from AM controls in all the shoot mass-normalized RSA parameters tested, showing an averaged 1.4- and 2.0-fold induction in the specific RL (sRL), sRSA, sRV, and sRT of mycorrhizal MtSucS1-as12 and -as19, respectively (Fig. 2C). Thus, both antisense lines increased their relative production of root tissue under AM conditions, while their non-symbiotic shoot mass-normalized root system growth ranged near control levels in line as19 and slightly decreased in as12 under full supplementation (Supplemental Table S1D). Based on the diverse physiological data presented, we conclude that an MtSucS1 reduction substantially affects plant growth merely under AM-dependent, P\textsubscript{i}-limited conditions.

Antisensing MtSucS1 Leads to Reduced P and N Contents in Mycorrhizal Plants

Cellular pools of mobile cytoplasmic and vacuolar orthophosphates were measured to evaluate the P\textsubscript{i} uptake efficiency. The P contents of the root and shoot tissues of all AMF-colonized lines were elevated compared with NM tissues, but mycorrhizal MtSucS1-as material contained considerably less P than equally treated controls (Fig. 2D). It is worth noting that P levels in shoot samples excluded the particularly P\textsubscript{i}-demanding pods (Bryla and Koide, 1990), which were also substantially reduced in both size and number in the mycorrhizal MtSucS1-as lines (Fig. 2B). Along with the least P concentration in the aboveground material (data not shown) and a 1.33-fold lower dry weight of the MtSucS1-as12 shoot, there was a markedly decreased net P content of the shoot in this line. Together with substantial reductions on the root P pools, this also resulted in the lowest net plant P content for mycorrhizal antisense line as12 (Fig. 2D). Considering withdrawal of P from early-senescing leaves, the P concentration of the shoots of mycorrhizal antisense lines reached almost the reference level at 50 dpi but was maintained at the substantial expense of the major P storage in plant root tissue. By contrast, AM controls readily acquired sufficient P levels for the aboveground material without a withdrawal of P via augmented leaf senescence, lowered yields or seed filling (Fig. 2B), or reducing the P concentration and content in the root tissue (Fig. 2D).

Aboveground (generative organs excluded) and belowground tissues showed furthermore significantly reduced N contents (1.27- and 1.21-fold; Fig. 2D), causing a consistently increased C:N balance in both mycorrhizal MtSucS1-reduced lines. Taking the dry weight of the shoot into account, the aboveground N contents were 1.33- and 1.15-fold reduced in MtSucS1-as lines as12 and as19, respectively (Fig. 2D), also resulting in a significantly reduced whole-plant N (~1.18 and ~1.30). Deduced from C and N concentrations measured, the construction costs of mycorrhizal
roots (C demand per unit of root produced) were significantly reduced in MtSucS1-as lines (−1.09 and −1.11 in MtSucS1-as12 and -as19, respectively; Fig. 2D), presumably pointing to a reduced AM formation in MtSucS1-as lines. Together, these findings indicate a negative effect of MtSucS1 reduction on the P and N nutritional status of the shoot and root tissues under AM-dependent conditions.

MiSucS1 Reduction Is Accompanied by a Down-Regulation of AM Marker Genes

Since the development of an efficient AM considerably alters epidermal and cortical root cells as well as the overall root physiology (Bonfante, 2001; Genre et al., 2008; Guether et al., 2009; Pumplin and Harrison, 2009), effects of MtSucS1 reduction were expected to be mirrored in the root transcriptome. We examined transcriptional changes of 36 M. truncatula and 12 G. mosseae genes with AMF colonization (primer sets in Supplemental Table S3). Particular attention was paid to host genes primarily coding for AM-specific markers with special focus on arbuscule-related genes (Hohnjec et al., 2005, 2006; Liu et al., 2007; Gomez et al., 2009). Regarding glycoside hydrolases, we focused on the SucS family (Hohnjec et al., 1999), several Inv, as well as fructan-active enzymes (1-FEH; Wiemken et al., 1986). Glomus candidate genes had assigned functions during fungal symbiotic life.

As expected, antisensing MtSucS1 significantly reduced the MtSucS1 mRNA levels in as19 and as12 mycorrhiza (20% and 13% of control levels, respectively; Table I). Subsequently, both lines displayed highly similar transcriptional changes for the candidate genes checked. As compared with mycorrhizal controls, differential transcription (2-fold threshold; P < 0.05) was monitored for 13 out of 29 plant and 10 out of 12 G. mosseae genes in at least one type of the MtSucS1-altered mycorrhiza. Common repressions were especially apparent for the set of AM marker genes.

**Figure 2.** Growth and developmental characteristics of MtSucS1-as lines. A, Leaf number development in mycorrhizal MtSucS1-as and control lines. B, Relative vegetative and reproductive characteristics of mycorrhizal MtSucS1-as and control lines at 50 dpi (n = 30 individuals per line). DW, Dry weight; FW, fresh weight; RWR, root weight ratio as DW_root per DW_plant. C, Specific root parameters of mycorrhizal MtSucS1-as and control lines. The specific root length, surface area, root volume, average root diameter, and root tip number of mycorrhizal MtSucS1-as and control lines were expressed as root parameter per unit of shoot dry weight (DW_root) at 50 dpi (σ = 9, n = 16 mycorrhizal plants). D, Root and shoot C, N, and P contents of mycorrhizal MtSucS1-as and control lines. The mineral contents were calculated per unit dry weight in 50 dpi-old mycorrhizae (n = 8 biological replicates per line). Significantly different means ± SE are indicated as follows: a 0.05 > P ≥ 0.01, b P < 0.01. Relative values are in comparison with control levels set to 100%. Gray bars, AM MtSucS1-as line as12; dark gray bars, AM MtSucS1-as line as19; white bars, AM control lines; gray squares and dashed gray line, NM MtSucS1-as; white circles and dashed black line, NM controls. Yield indicates number of pods at day of harvest. cc, Construction cost; % c, Percentage in relation to controls.
Table 1. Ratios of relative gene expression in MtSucS1−as lines at 50 dpi

Relative expression values were calculated via MtTef1a normalization. Tentative consensus (TC), EST, and gene sequences are sorted via x-fold changes with respect to as19. Correlation analyses of genes to MtSucS1 expression are indicated by +++ (r ≥ +0.6), ++ (+0.6 > r > +0.4), + (+0.4 ≥ r > 0), and − (0 ≥ r ≤ −0.4). Relative expression for as19 and as12 denotes mean transcript ratios of MtSucS1−as lines versus control lines. Relative expression ratios exceeding the criteria of significance of both 2-fold threshold and P < 0.05 are shown in italic boldface. Ratios achieving only one criterion are marked in boldface. Columns 5 and 6 display transcript ratios of MtSucS1−as lines versus control lines per MtPT4/CmtTe1 activity factor. This activity factor correlates candidate gene expression with the number of active arbuscules (measured via MtPT4) and the total presence of the AMF in the colonized root (measured via GmTe1). Means and ratios were based on five biological replicates. *, Significant difference of 0.05 > P ≥ 0.01. **, Significant difference of P < 0.01. na, Not applicable; nd, not detected.

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genes (MtBcp1, MtScp1, MtPT4, MtGst1, MtAnn2, MtDxs2, MtHai1, and MtGlp) and all fungal genes measured (Table I). Particularly, the AM-specific MtPT4 expression (Harrison et al., 2002) was markedly reduced to 22% and 24% of control levels in as19 and as12, respectively (Table I), indicating extensive alterations in a reliable parameter for AM functionality and arbuscule vitality (Isayenkov et al., 2004; Javot et al., 2007).

Transcripts of several Suc-hydrolyzing enzymes such as the CW Inv and vacuolar acid Inv (TC107108 and TC96236) were clearly reduced in both MtSucS1-as line mycorrhizae, while significant repressions of the CW Inv I and II (BG148180, TC103706, and TC98116), the neutral Inv (TC106499), and the neutral/alkaline (N/A) Inv (TC99238 and TC106886) were found in one antisense line, preferentially in the stronger reduced line as12 (Table I). Differential transcriptional responses in MtSucS1-as12 and -as19 mycorrhizae were evident for the 1-FEH genes (Table I), possibly pointing to their tight dependency on the CH status of the mycorrhizal root tissue.

All fungal transcripts decreased sharply in MtSucS1-as mycorrhizae, with the effect being more pronounced in the strongly MtSucS1-reduced line as12. Constitutive fungal genes (GmTef1, GmITS12, Gm28sRNA; Isayenkov et al., 2004) were consistently reduced more than 2-fold in both MtSucS1-as AM lines, with GmTef1 being the most constant housekeeping gene. Using GmTef1 expression levels, endophyte concentrations in symbiotic roots diminished by 63% in as12 and by 59% in as19. This coincided with significant transcriptional decreases in six out of 12 G. mosseae genes, suggesting an MtSucS1-dependent mycosymbiotic transcription (Table I). In addition, transcripts of GmPMA1 and genes involved in fungal storage processes (e.g. GmGlyS and GmAEL0) were significantly reduced at least in MtSucS1-as12 (Table I).

Applying the Bravais-Pearson coefficient of correlation r, the expression of several plant and fungal genes correlated positively with MtSucS1 transcript levels (Table I). Notably, a particularly strong correlation (rMtSucS1 ≥ 0.6) was found for plant AM marker genes (e.g. MtScp1, MtAnn2, MtPT4, MtGlp1, MtHai1, MtGst1, and MtDxs2; Table I), indicating a tight association of AM-related transcript levels with the relative MtSucS1 expression. The transcription of the 1-FEHs (TC96754 and TC109927), an N/A Inv (TC106886; Table I), and the (H+)/hexose cotransporter MtSt1 (Table I) solely correlated negatively with the MtSucS1 expression. In the case of mycosymbiont candidate gene expression, all fungal transcripts exhibited a positive interrelation with the level of MtSucS1 expression, with particularly strong correlations for GmICL, GmHAS, GmAEL0, and GmTef1 (Table I).

Since the significantly altered expression patterns in MtSucS1-limited mycorrhizae coincided with marked decreases in the fungal presence in mycorrhizal tissues, candidate gene expression values were further processed for the following reasons. Although all plant lines were treated at the same time with the same amount of inoculum, differences in transcription might be due to technical variations such as minor alterations in spore numbers and quality, affecting the fundamentals of root colonization, or to intrinsic genetic effects of the MtSucS1-as expression, causing a delayed and altered mycorrhization of the CH-limited plants. Thus, to better evaluate the efficiency of mycorrhizal infection in the symbiotic lines, to consider different levels of fungal occupation, and to account for shifts in the proportion of young and mature arbuscules and their efficiency for the symbiotic nutrient exchange, we defined an “activity factor” that correlated candidate gene expression with the number of active arbuscules (measured via MtPT4) and the total presence of the AMF in the colonized root (measured via GmTef1). Hence, transcript levels that were additionally normalized to this activity factor (MtPT4/GmTef1) are more meaningful, since gene expression is put in relation to the presence of efficient, mature arbuscules per fungal unit, thus considering fungal presence and AM developmental stage after conventional normalization via the general plant housekeeping gene MtTef1α (Table I).

It is important to note here that the MtPT4/GmTef1 ratio of the mycorrhizal control roots averaged 2.51, whereas as19 and as12 achieved significantly reduced values of 1.07 and 0.77, already implying marked reductions in the number of functional arbuscules per unit of AMF. Despite a correction via the respective line-specific activity factor, the relative expression of MtPT4 additionally normalized in this way remained at a markedly reduced level in MtSucS1-as mycorrhizae when compared with mycorrhizal controls, attaining −1.8 in MtSucS1-as19 and −1.6 in MtSucS1-as12 (Fig. 3A; Table I). The MtPT4/GmTef1-normalized values showed consistent repressions for MtSucS1 and MtBcp1 in MtSucS1-as mycorrhiza (Fig. 3A). Trends toward reduced transcriptional levels in at least one type of SucS1-reduced mycorrhiza were also monitored for the AM marker MtScp1 (Fig. 3A). Since MtBcp1, MtPT4, and MtScp1 represent well-studied AM marker genes being most active in the mature arbuscule phase, their strongly reduced expression levels in the antisense lines could point to a relevance of MtSucS1 for the development and maintenance of mature plant/fungus interfaces.

The expression profiles of the remaining plant genes were subclassified into two clusters, applying a 2-fold threshold after normalization via the MtPT4/GmTef1 unit (Fig. 3, sixth column). The AM marker genes MtGlp1, MtDxs2, MtHai1, MtAnn2, and MtGst1 achieved expression levels between +1.4- and +1.7-fold in mycorrhizal as19 and between +2.1- and +3.5-fold in as12 (Fig. 3B). The genes activated more than 2-fold per active symbiotic interface primarily encoded Suc-degrading enzymes (Fig. 3C). In fact, MtSucS1 reduction appeared to up-regulate the arbuscule-related expression of genes encoding cytosolic glycoside hy-
drolases, since mRNAs from N/A Inv and neutral Inv genes showed tendencies toward an enhanced expression (Fig. 3C). Highest relative transcription in mycorrhizal MtSucS1-as root extracts was found for genes encoding 1-FEH (TC109927 and TC100757), N/A Inv (TC106886 and TC106499), and the vacuolar Inv PsI-1. Moderate reciprocal responses to MtSucS1 reduction in AM tissues also denote the relative inductions observed for MtSucS2 and MtSucS3 (Fig. 3B) as well as for MtSucS5 and MtSucS/MtSucS4 (Fig. 3C). Together, the up-regulation of genes from clusters B and C indicates an attempt of the macrosymbiont to compensate for the MtSucS1 knockdown to some extent. By contrast, since the activity of the complete set of fungal genes per active arbuscule remained clearly repressed in MtSucS1-reduced mycorrhizae (Fig. 3D), the microsymbiont transcription appears to be MtSucS1 dependent to a high degree.

MtSucS1 Reduction Is Correlated with Changes in Steady-State Pools of Polar Metabolites in AM Roots

The physiological impact of endophyte infection under MtSucS1 reduction was analyzed, monitoring differential abundances of 98 metabolic compounds in MtSucS1-as and control mycorrhizae (Supplemental Table S4). A comparison of the metabolite compositions displayed considerable alterations in 61 substances. The primary metabolic pools were mainly affected by MtSucS1 limitation, accumulating at -6.0- to +2.6-fold different levels in antisense line root extracts. Eighty-five percent of the metabolites tested, representing important nodes of the glycolysis, the tricarboxylic acid cycle, and the amino acid biosynthesis, appeared to be conjointly altered and preferentially reduced in MtSucS1-as mycorrhizae (Supplemental Table S4).

Accumulated Suc levels reciprocally correlated with the extent of MtSucS1 expression (1.40- and 1.46-fold...
increased in as19 and as12, respectively; subsequently +1.40/+1.46), indicating reduced sink strength in, but also a sufficient Suc supply to, the MtSucS1-altered mycorrhizae. Subsequently, the major hexoses and immediate Suc cleavage products Glc (−1.20/−1.08) and Fru (−1.24/+1.08) tended toward lower abundances in MtSucS1-as extracts. Moreover, MtSucS1-reduced mycorrhizae were associated with significant decreases in the hexose/Suc ratio, yielding 68% of control levels in as12 and 62% in as19. Concerning the soluble sugar content, pool sizes were either concomitantly reduced as for cellulose, trehalose, and raffinose or enhanced as for maltose, Gal, rhamnose, and melibiose. The gross of sugar acids derived from Glc such as glyc erate or glucuronate as well as dicarboxylic or hydroxy acids such as maleate and 2-hydroxyglutarate decreased in symbiotic MtSucS1-altered roots. The abundance of the endofungal, nonmetabolizable sugar alcohol mannotol (Rasmussen et al., 2008) was clearly reduced in both MtSucS1-as AM extracts (−2.30/−2.31). According to the reduced tissue P content in MtSucS1-as material (Fig. 2D), the phosphorylated metabolic pool also markedly decreased with an MtSucS1 knockdown (−1.40/−1.35). Beyond that, with direct linkage to a functional AM, the fungal C pool trehalose (−1.43/−1.72) as well as the plant N storage and transport compound Asn (−6.00/−3.65) were significantly reduced in MtSucS1-as mycorrhizae. With respect to fungus-mediated biotic stress or AM-mediated plant protection (Conrath et al., 2006; Pozo et al., 2009), the levels of γ-aminobutyrate (−2.33/−1.78) and the classical defense-related plant compound salicylic acid (−1.12/−1.38) also showed tendencies for reduction in mycorrhizal MtSucS1-as extracts, together indicating a reduced establishment and performance of AM in the MtSucS1-limited root material.

Glc reductions were accompanied by lowered pool sizes of almost all glycolytic intermediates, including Glc-6-P, Fru-6-P, dihydroyacetone phosphate, 3- and 2-phosphoglycetate, and phosphoenolpyruvate, which implies a reduced catabolic activity in the glycolytic payoff phase upon MtSuc1 knockdown. Subsequently, compounds involved in the tricarboxylic acid cycle such as pyruvate, α-ketoglutarate, and succinate were reduced. Citrate, cis-aconitate, isocitrate, and malate were considerably more abundant in MtSucS1-as mycorrhiza (average +1.31 in as19 and +1.25 in as12), suggesting an adjusted glyoxylate pathway under conditions of limited Suc breakdown.

Marked reductions were found in the content of 13 out of the 20 proteinogenic amino acids, being most apparent for Asn. Reduced pool sizes were prominent for the major amino acids Ala, Arg, Asp, Lys, Ser, Thr, and Tyr, exhibiting average metabolic changes of −1.86 in as19 and −1.44 in as12. The minor amino acids Cys, Gly, Ile, Lys, Pro, Tyr, Trp, and Val, the aromatic amino acid precursor shikimate, as well as several intermediates and derivatives of the amino acid biosynthesis were also reduced in both antisense lines (Supplemental Table S4). By contrast, Glu and Gln showed average 1.3-fold increases in MtSucS1-as mycorrhiza. Nevertheless, along with approximately 1.2- to 1.3-fold reduced N concentrations and contents of mycorrhizal MtSucS1-as roots (Fig. 2D), the summarized content of all proteinogenic amino acids in line as19 comprised 74% and that in line as12 81% of control levels. Furthermore, N-containing metabolites representing the urea cycle as well as uncommon and nonproteinogenic amino acids (e.g. pyroglutamate and β-Ala) showed marked reductions. The latter is in agreement with likewise repressed pantethenate, the essential precursor of CoA (Raman and Rathinasabapathi, 2004). Intriguingly, classical markers of senescence processes such as RNA degradation are increased, since the free nucleobases adenine, cytosine, uracil, and thymine accumulated in MtSucS1-as mycorrhizae (average +1.22/+1.24), in concert with an enhanced level of Rib at least in line as12.

In conclusion, several molecular symbiosis markers such as the AM-related plant and fungal transcriptional and important nodes of the C, P, and N metabolism of mycorrhizal roots were significantly altered by an MtSucS1 reduction.

Antisensing MtSucS1 Leads to a Higher Proportion of Senescing Arbuscules

Although at 50 dpi the MtSucS1-as and control lines displayed Arum-type mycorrhizae with G. mosseae, a detailed investigation revealed a considerably lower mycorrhizal status with a reduced internal colonization upon MtSucS1 reduction. Controls achieved a uniform root length colonization (RLC) of 49.8%, whereas RLC reached 37.4% in line as19 and 38.8% in line as12 (Fig. 4A). Moreover, clear differences in the colonization pattern were found in transverse sections within a given colonized region. Independent of the total colonization, the successive fungal progression within the root cortex differed significantly, evidenced by densities of the intraradical hyphae, arbuscule, and vesicle colonization per root intersect. In fact, these intracellular fungal structures appeared 40% to 60% less numerous in MtSucS1-reduced mycorrhizae. In MtSucS1-as19, hyphal densities were less than in as12 and controls, coinciding with rarely produced vesicles (Fig. 4A), an effect most likely related to the enhanced root system partitioning. Despite an increased relative root system growth in MtSucS1-as plants (Fig. 2, B and C), the total amount of root length colonized by the fungus never exceeded the AMF occupancy observed in control mycorrhizae. Moreover, the specific root colonization (accentuated value of AMF per total root fresh weight) showed significant 1.52- and 1.55-fold reductions in as12 and as19, respectively (Fig. 4A). In general, using 35 individually analyzed mycorrhizae formed by the different plant lines, a positive correlation of the visually estimated RLC and parameters of plant fitness (e.g. generative yield and fresh weight of the shoot; data not shown) were evident. Furthermore,
MtSucS1 expression and transcripts of plant AM marker genes strongly correlated with the reduced colonization ($0.49 < r_{RLC} < 0.74$), an effect most prominent for MtPT4.

In a separate approach, RLC of MtSucS1-as19 and control mycorrhizae was monitored in a time course up to 81 dpi (Fig. 4B). Mycelium was visible on and inside control roots at 16 dpi. Intercellular and intracellular hyphal growth was not yet spacious, coinciding with some young arbuscules in cortical cells. Data from regular harvests exhibited the establishment of a mature reference AM within 36 dpi. The progression of the RLC in controls reached a plateau level of 65% to 70%, starting at 53 dpi (Fig. 4B) with numerous well-developed arbuscules, which were by now either mature or already degenerating (Fig. 4L). By contrast, AM development in MtSucS1-as19 was initially delayed and proceeded slower, resulting in a lower plateau than controls by more than 60 dpi. Until the end of the experimental period at 81 dpi, the mycorrhiza status of line as19 remained at an approximately $1.54$-fold proportion of the RLC observed in reference mycorrhiza.

Confocal laser-scanning microscopy of fluorescence-labeled fungal structures demonstrated significant alterations in the morphology and life span of arbuscules in both mycorrhizal antisense lines. Here, a sharp reduction in the total number and proportion of mature arbuscules was evident (Fig. 4, C–K). In mycorrhizal controls, the distribution of the stages young, mature, degenerating, and dead arbuscules (Floß et al., 2008) was 1:2.3:1:4:1.1 (Fig. 4L), depicting a successive colonization process with balanced rates of arbuscule decay and reemergence. In contrast, MtSucS1-altered lines exhibited a significantly different proportioning.

**Figure 4.** Mycorrhiza phenotypes of MtSucS1-as and control lines. A, *G. mosseae* root length colonization and specific internal colonization of intraradical hyphae, arbuscules, and vesicles in mycorrhizal regions at 50 dpi. B, Root length colonization time course of mycorrhizal MtSucS1-as19 and control lines as revealed from sequentially harvested plants. Each point represents the mean ± se of at least three plants. Regression curves for mycorrhizal lines are shown. C to K, Representative fluorescence images of AlexaFluor488-stained mycorrhizal root segments at 50 dpi. C to E, MtSucS1-as12 (C), MtSucS1-as19 (D), and control lines (E) depicting the significantly reduced mycorrhiza status in both MtSucS1-as lines. F to H, Pronounced arbuscule senescence in MtSucS1-as12 and -as19 segments displayed by high proportions of degenerating (dgA) and dead (deA) arbuscules. Bars = 75 μm (F–H) and 30 μm (I–K). L, Distribution of the arbuscule developmental stages in MtSucS1-as lines at 50 dpi compared with controls set to 100%. Significantly different means ± se are indicated as follows: * $0.05 > P > 0.01$, $^b P < 0.01$. Gray bars, MtSucS1-as line as12; dark gray bars, MtSucS1-as line as19; white bars, controls; gray squares and dashed gray line, mycorrhizal MtSucS1-as19; white circles and solid line, controls. Relevant morphological fungal structures such as intraradical hyphae (H), arbuscules (A), and septae (S) are highlighted with arrowheads. AC, Arbuscule colonization; HC, intraradical hyphae; mA, mature arbuscule; % c, percentage in relation to controls; VC, vesicle.
DISCUSSION

The key result of our study is a significantly affected internal colonization of mycorrhizal MtSucS1-reduced plants. Here, the generally highly branched intraradical hyphal network is less differentiated, accompanied by alterations in fungal root occupancy, the timing of infection, fungal development, and, most strikingly, in the longevity of the asynchronous AM symbiosis. This arbuscule phenotype matches observations for M. truncatula plants depleted in the AM marker genes MtPT4 (Javot et al., 2007) and MtDxs2 (Floß et al., 2008). Since during nodulation, MtSucS1-as plants also exhibited early symbiosome senescence (Baier et al., 2007), MtSucS1 is likely involved in the C supply to microsymbionts and in the sufficient development of endosymbiotic interfaces in general.

The defective internal mycorrhization phenotype of MtSucS1-reduced plants implies a distinct symbiotic function for an MtSucS1-dependent Suc breakdown, serving various metabolic purposes particularly in the arbuscule-containing and -adjacent cells in addition to the known function of apoplastic Inv for fungal hexose supply (Schaarschmidt et al., 2007a). Along with an impaired symbiotic Suc utilization in MtSucS1-as AM, several lines of evidence point to a diminished C transfer to the AMF. Marked reductions of the main Glomus hyphal biomass located inside the root (Maherali and Klironomos, 2007) indicate a generally hindered total fungal growth and an insufficient C utilization for the mycosymbiont in MtSucS1-as AM. Moreover, the strongly reduced fungal structures (particularly fewer vesicles) and characteristic interim fungal C pools linked to a functional AM (trehalose and mannitol) as well as the repression of the glycerogen and fatty acid synthesis clearly highlight fungal C limitations. In addition, reduced mycorrhiza construction costs mirrored an impaired symbiosis formation and maintenance, since the extraradical mycelium and frequently developed spores directly contribute to the C budget of the plant (Peng et al., 1993; Olsson and Johansen, 2000). Comparable colonization and C starvation phenotypes were observed in Inv-modified mycorrhizal tobacco, deficient in the direct fungal hexose supply that was altered either by the expression of an apoplastic Inv inhibitor under the control of an inducible promoter (Schaarschmidt et al., 2007a) or by the activity of a p35S-driven apoplastic Inv in leaves (Schaarschmidt et al., 2007b). The phenotypes recorded resembled plants with reduced Suc translocation to AM due to an impaired photoassimilation (Tester et al., 1985; Son and Smith, 1988; Vierheilig et al., 2002), one factor also reducing the proportion of functional arbuscules (Hayman, 1974). Similarly, the incomplete and delayed fungal colonization demonstrates an effect on AM formation, maintenance, and efficiency by a limited MtSucS1-mediated C availability at plant/fungus interfaces. Extending the model that merely apoplastic Inv activity increases apoplastic sugars during AM (Bago et al., 2000, Schaarschmidt et al., 2006), our findings support the assumption that cytoplasmic MtSucS1 participates in C efflux toward the arbuscule apoplast. It should be noted here that the Suc-dependent Suc breakdown requires half the net energy of the Inv-mediated pathway, allowing SucS to operate under conditions of lower oxygen (Koch, 2004). Hence, cytoplasmic MtSucS1 could meet the CH requirements during AM at times of high glycolytic and C demands (e.g. under increasing anaerobiosis in maturing arbuscule-containing cells). MtSucS1 expression in these and in arbuscule-adjacent cells (Hohnjec et al., 2003), in concert with a symplastic cell-to-cell movement of Suc cleavage products and/or an AM-induced expression of hexose transporters in cortical cells of colonized regions, could support the enhanced plant and fungal metabolism in active arbuscules. Notably, hexose transporter genes were induced in MtSucS1-as mycorrhizae (e.g. MtSt1; Harrison, 1996). Together with the enhanced expression of genes encoding N/A Inv as well as other Suc- and fructan-hydrolyzing enzymes, the up-regulation of hexose transporter genes could thus sustain a basic hexose supply to MtSucS1-as arbuscules.

Interestingly, we observed limited increases of symbiotic root Suc pools in antisense lines compared with controls, while the levels of hexoses were hardly changed. Given that the metabolic pathways by which C is handled in AM underlie multilevel regulations (Bago et al., 2000) and that Suc breakdown is also essential for the initiation of hexose-based sugar signals in Suc-importing structures (Koch, 2004), MtSucS1-reduction could cause posttranscriptional and posttranslational regulatory steps that affect MtSucS1 activity. Apart from development-, tissue-, and cell-specific transcriptional control (Fu and Park, 1995; Zrenner et al., 1995; Barratt et al., 2001), SucS expression is modulated by various stimuli such as the tissue CH status (Koch et al., 1992), environmental conditions or anaerobiosis (Shaw et al., 1994), while the steady-state level of SucS is posttranscriptionally regulated via the cellular redox state (Marino et al., 2008). Considering a significantly reduced MtSucS1
expression in antisense mycorrhizae, posttranslational regulations, such as by reversible serine-phosphorylation (Carlson and Chourey, 1996; Winter et al., 1997, 1998; Wiemken et al., 2008), represent possible strategies to sustain residual MtSucS1 activity in colonized antisense roots. Additionally, a variety of regulatory stimuli and mechanisms to modulate Inv at the transcriptional and posttranscriptional level (Sturm, 1999; Roitsch and Ehne, 2000; Roitsch et al., 2003; Koch, 2004) could further have a compensatory impact on Suc utilization in mycorrhizal antisense roots. Nevertheless, the phenotypic traits displayed by our mycorrhizal antisense lines suggest that the above-mentioned possibilities to bypass hexose limitations were apparently not sufficient to allow the formation of a more complex hyphal network in antisense roots and, moreover, to support the establishment of normal numbers of mature arbuscules in colonized regions under MtSucS1 limitation.

Most likely, SucS and Inv act synergistically in AM. Their concerted activities were particularly evident during the highly C-demanding AM initiation (Schaarschmidt and Hause, 2008). For the final AM stages, when arbuscules are purged from root cells, Blee and Anderson (2002) postulated that the actual SucS function is related to the reestablishment of precolonization conditions (e.g. the synthesis of starch and cellulose). In addition, they assumed a regulation of arbuscule formation and location in cortical cells by C availability (Blee and Anderson, 1998). In MtSucS1-altered mycorrhizae, the formation and proportion of mature arbuscules were indeed reduced, coinciding with rising numbers of degenerating and dead arbuscules. This points to a degradation of insufficiently formed, presumably less functional arbuscules prior to their maturation, an effect that was also observed for transgenic lines where only the apoplastic Suc metabolism was manipulated (Schaarschmidt et al., 2007a, 2007b).

One drawback of visual colonization estimations is that these include both living and dead fungal material and largely exclude the plasticity of functional mycorrhizae (Burleigh et al., 2002). The substantial repression of Glomus and plant AM marker genes observed here, on the other hand, represent principal measures of a reduced functional colonization intensity and symbiotic performance, resulting from lower colonization of MtSucS1-altered roots by living fungal biomass. Moreover, our attempt to evaluate AM marker gene expression in relation to the presence of functional, mature arbuscules revealed substantial down-regulation, particularly for MtBcp1, MtPT4, MtScp1, and MtHa1 (Harrison et al., 2002; Krajinski et al., 2002; Liu et al., 2003; Hohnjec et al., 2005). This points to reductions of the encoded proteins that most likely function in the periarbuscular membrane itself or in the case of MtBcp1 in the periarbuscular membrane domain surrounding arbuscule trunks (Pumplin and Harrison, 2009), again indicating that arbuscules were declining in their development and function under MtSucS1 limitation.

The multistep processes of fungal invasion and, in particular, arbuscule accommodation, accompanied by massive reorganization of the host cell morphology involving intracellular structures (Bonfante and Perotto, 1995; Genre et al., 2005), might constitute additional explanations for the effect of MtSucS1 reduction. Several studies demonstrated profound cytoskeletal rearrangements before hyphae invade cortical cells (Genre et al., 2005) and from the early stages of arbuscule development until the formation of senescing arbuscules (Genre and Bonfante, 1998; Blancaflor et al., 2001). In addition, reassembly of actin filaments supported organelle positioning near the periarbuscular membrane (Genre and Bonfante, 1998; Lohse et al., 2005; Pumplin and Harrison, 2009). Apart from these extensive cytoskeleton reorganizations, the differentiation and de novo synthesis of perifungal plasma membrane and CW extracellular matrix material from the beginning of AM colonization until the branching hyphae in arbuscule-containing cells (Bonfante-Fasolo, 1984; Balestrini et al., 1996; Balestrini and Bonfante, 2005) are major and costly events, possibly involving an MtSucS1-mediated Suc breakdown already during the earliest phases of AM formation.

Apart from the possibility that MtSucS1 reduction could affect intraradical fungal growth already prior to arbuscule formation, our arbuscule-related phenotype points to the importance of MtSucS1 for the accommodation of fungal structures via transient cytoskeletal rearrangements in arbuscule-containing and -adjacent cells as well as membrane deposition and/or cellulose synthesis relevant for invading hyphae. Moreover, the limited but frequently replicated cycle of arbuscule growth, septation, collapse, and senescence in successive fungal colonizations (Alexander et al., 1989; Javot et al., 2007) could impose a repeated need for a continuous MtSucS1-mediated C supply, contributing to the activated metabolism of nascent arbuscules. Consequently, the reduced colonization in MtSucS1-altered plants, the lower and occasionally arrested arbuscule formation, as well as their premature degeneration can also be interpreted as a secondary effect of an MtSucS1 knockdown that results from a decreased plant/fungus contact area.

In conclusion, wild-type MtSucS1 levels apparently are directly or indirectly prerequisite not to induce but to sustain normal arbuscule maturation and lifetime. Although arbuscules are initiated under MtSucS1 reduction, they cannot properly mature, indicating that Inv cannot fully substitute for SucS during later stages of arbuscule development.

MATERIALS AND METHODS

Plant Material, Conditions of Growth, and Fungal Inoculation

Two independent Medicago truncatula p35S-MtSucS1-as lines (as12 and as19) were compared with two independently transformed, homozygous control lines expressing pMtSucS1-gusAint fusions (Hohnjec et al., 2003; Baier
et al., 2007). Seeds were surface sterilized, scarified (Baier et al., 2007), initially grown in 27 cm² pots, and fertilized with half-strength Hoagland solution twice a week containing 50 μmol P, until at least two trifoliate leaves developed. Afterward, plantlets were transferred into 600 cm³ pots, inoculated with a defined quantity of a granule inoculum (Glomus mosseae BEG12; Biorize R&D), and supplemented with half-strength Hoagland solution twice a week. Growth conditions were set to a 16-h-light (24°C)/8-h-dark (18°C) cycle, providing a photosynthetically active radiation of 500 μm²·s⁻¹ at 60% relative humidity.

Plant Phenotyping, Tissue Nutrient Content Analyses, and Metabolite Profiling

Measurements of aboveground development, photosynthetic performance, root parameters, and the gas chromatography-mass spectrometry-based profiling of polar metabolites were described by Baier et al. (2007). Plant material was harvested in the middle of the light cycle at 50 dpi. Upon microscopy and dry weight measurements, aliquots of 1- to 2-cm-long root subsamples were separated. Remaining mycorrhizal root material was immediately frozen in liquid N₂ and stored at −80°C. MGR were obtained as MGR = 100 × [(AM – NM)/NM], using the shoot dry weights of individual AM plants and the mean shoot dry weight of corresponding NM lines (Smith et al., 2003). The scaling of biomass relationships [dry weight of shoot (g) and dry weight of root (g)] assessed grade shifts on allometry. Using log₁₀ y = k log₁₀ x, the slope of the best-fit linear regression defines the allometric coefficient k. Tissue construction costs [mmol C g⁻¹ dry weight] were calculated according to Mortimer et al. (2008). RSR were based on fresh weights. Root weight ratios were measured as dry weight of root per dry weight of plant. Dry weight of root was calculated via line-specific dry weight/fresh weight ratios, based on individual root subsamples. Dry matter was obtained by freeze or oven drying (70°C). Mineral contents were calculated per unit of dry weight of 50-dpi mycorrhizal plants. N and C contents were measured via gas chromatography as described by Baier et al. (2007). Using gas chromatography-mass spectrometry, P accumulation was determined via integration of the peak area of the characteristic fragment ion of mass-charge ratio 299 according to Schliemann et al. (2008).

Total RNA Isolation and Quantitative RT-PCR

Mycorrhiza samples were taken from frozen stocks, pooled, and ground in an MM100 mixer mill (Retsch) prior to metallization and RNA extractions. Total RNA isolation and DNase I on-column digestion were performed according to the manufacturer’s instructions (Qiagen). RNA preparations were electrophoretically and spectrophotometrically checked for yield and quality (NanoDrop ND-1000; NanoDrop Technologies). Applying the Quant iTect Reverse Transcription kit (Qiagen) and 500 ng of total template RNA (final volume = 20 μl), first-strand cDNA synthesis was performed, according to the supplier’s protocol. Via in silico analyses of the M. truncatula Gene Index (http://compbio.dfc.umd.edu/), tentative consensus sequences, representing genes for AM or sink metabolism, were selected. The unique, gene-specific PCR primer sets (Supplemental Table S3) displayed the following parameters:

- Polymerase chain reaction (PCR) primer sets (Supplemental Table S3) displayed the following parameters:
  - Melting temperature of 53 °C,
  - Length and melting behavior of the PCR product were analyzed.
- RT-PCR was performed with the Quant i Tect SYBR Green PCR Kit (Qiagen), using 1/20 volume of first-strand cDNA derived from 25 ng of total RNA (final volume = 25 μl).
- Glomus genes were profiled in a one-step RT-PCR apparatus using the Quantace SensiMix One-Step Kit (Quantace).
- Expression results were averaged over six biological and two technical replicates using the constitutive translational elongation factor gene MtTEf1a (TC104858) for internal normalization.
- The CT_MtTef1a was analyzed at least twice per run, and average values were used to calculate relative gene expression levels (2⁻ΔCT; ΔCT = CT_expr – CT_MtTef1a).

Mycorrhizal Assessment

Light microscopy was performed on cleared, Shaeffer ink-stained root fragments (Vierheilig et al., 1998) using an Olympus BH-2 light microscope with an Olympus C-2000Z digital camera (Olympus Optical). RLC was assessed by the grid line intersect method (Giovannetti and Mosse, 1980) using a Zeiss stereomicroscope (Carl Zeiss) recording more than 1,000 intersects per pooled biological replicate. Hyphal, arbuscular, and vesicular colonization were determined in colonized regions by the magnified (200×) line intersect method (McConigle et al., 1990). Arbuscule developmental stages (Filot et al., 2008) were studied on eight samples per transgenic line on WGA-Alexa Fluor488-stained root segments (Javot et al., 2007) by a Leica SP2 laser-scanning microscope (Leica Microsystems) recording more than 190 arbuscules per biological replicate (c, 2,160; as12, 1,800; as19, 1,900 arbuscules). Image processing was carried out with Adobe Photoshop 7.0 (Adobe Systems) and Leica LCSLight.

Statistical Analyses

Parameters/variables were calculated as means ± se and analyzed for significance by Student’s t test with the following P levels: a 0.05 > P ≥ 0.01; b P < 0.01. Coefficients of Bravais-Pearson product-moment correlation r = 0.61 were defined as strong, and 0.31 > r < 0.61 were defined as weak. Critical r values depended on the independent replicate number n. All statistical tools used were incorporated in MS Excel 2007 (Microsoft).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Attributes of the physiology fitness of MtSucS1-as and control lines cultured under nonsymbiotic, Pᵢ-deprived, and fully supplemented conditions compared to growth patterns observed under mycorrhizal supplemented conditions.

Supplemental Figure S2. Photochemical yield of MtSucS1-as and control lines at a mature mycorrhizal stage (28 dpi).

Supplemental Table S1. Phenotypic variables of nonsymbiotic MtSucS1-as and corresponding control lines cultured under NM, Pᵢ-deprived, or fully supplemented growth conditions.

Supplemental Table S2. Leaf developmental attributes of mycorrhizal MtSucS1-as and control lines.

Supplemental Table S3. Primer sequences used in real-time RT-PCR experiments on total RNA derived from mycorrhizal root tissue.

Supplemental Table S4. Ratios of polar metabolite steady-state levels in MtSucS1-as and control lines at 50 dpi.

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LITERATURE CITED


