The *Medicago truncatula* Sucrose Synthase Gene *MtSucS1* Is Activated Both in the Infected Region of Root Nodules and in the Cortex of Roots Colonized by Arbuscular Mycorrhizal Fungi

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The *MtSucS1* gene encodes a sucrose synthase (EC 2.4.1.13) in the model legume *Medicago truncatula*. To determine the expression pattern of this gene in different organs and in particular during root endosymbioses, we transformed *M. truncatula* with specific regions of *MtSucS1* fused to the *gusAint* reporter gene. These fusions directed an induction to the vasculature of leaves, stems, and roots as well as to flowers, developing seeds, young pods, and germinating seedlings. In root nodules, strong promoter activity occurred in the infected cells of the nitrogen-fixing zone but was additionally observed in the meristematic region, the prefixing zone, and the inner cortex, including the vasculature. Concerning endomycorrhizal roots, the *MtSucS1* promoter mediated strongest expression in cortical cells harboring arbuscules. Specifically in highly colonized root sections, GUS-staining was furthermore detected in the surrounding cortical cells, irrespective of a direct contact with fungal structures. In accordance with the presence of an orthologous *PsSus1* gene, we observed a comparable regulation of *MtSucS1* expression in the grain legume *Pisum sativum* in response to microbial symbionts. Unlike other members of the *MtSucS* gene family, the presence of rhizobial or *Glomus* microsymbionts significantly altered and enhanced *MtSucS1* gene expression, leading us to propose that *MtSucS1* is involved in generating sink-strength, not only in root nodules but also in mycorrhizal roots.

Additional keywords: assimilate partitioning, phloem loading, transgenic *Medicago truncatula* plants.

In higher plants, assimilate partitioning is a fundamental process that describes the transport of carbohydrates from autotrophic source leaves to heterotrophic sink tissues. Sucrose, as

a stable disaccharide, is the main transport molecule for carbohydrates during the flow of metabolites to the sinks via phloem vessels (Avigad 1982; Hawker 1985). Upon phloem unloading and prior to utilization, sucrose has to be cleaved either by sucrose synthase (SucS, EC 2.4.1.13) or invertase (EC 3.2.1.26), both of them regarded as key enzymes responsible for carbon supply and sucrose partitioning in plants (Copeland 1990; Sturm and Tang 1999). Sucrose breakdown performed by SucS results in fructose and activated uridine-diphosphate (UDP)-glucose, which are precursors not only required for glycolysis but also important for cell wall (Hendrix 1990; Springer et al. 1986) as well as starch biosynthesis after conversion to adenosine-diphosphate (ADP)-glucose (Baroja-Fernández et al. 2001). Although sucrose degradation by SucS is reversible, these enzymes are predominantly detected in sink tissues, in which they carry out the cleavage reaction (Akazawa and Okamoto 1980). Generally, SucS activity is associated mainly with tissues in which cellulose and starch synthesis is favored (Baroja-Fernández et al. 2001; Chourey and Nelson 1976), but high levels of sucrose synthase gene (*SucS*) expression were also reported in phloem tissues of leaves, stems, and roots (Fu et al. 1995a and b).

Most plant species exhibit a small family of *SucS* genes. Due to their essential role in the control of assimilate partitioning from source to sink (Gibson 2000; Sturm and Tang 1999), *SucS* genes are highly regulated on the transcriptional and posttranscriptional level in a condition- or tissue-specific manner (Koch et al. 1992; Winter and Huber 2000). One mechanism of transcriptional regulation depends on a large leader intron in the 5' untranslated region (UTR), as was shown e.g., for *SucS* genes from *Solanum tuberosum* (Fu and Park 1995). In case of the *Zea mays shrunken-1* gene, this leader intron strongly stimulated gene expression in cell cultures and protoplasts (Clancy and Hannah 2002; Clancy et al. 1994). Furthermore, Fu and associates (1995a and b) demonstrated an increase of *Sus3* and a decrease of *Sus4* expression correlated with the presence of the 3' UTR.

A highly specialized sink tissue, in which at least one *SucS* isoform is strongly induced (Pawlowski 1997), is the root nodule, which is formed following a symbiotic interaction of gram negative bacteria of the family *Rhizobiaceae* with legumes. In return for an allocation of dicarboxylic acids, the plant benefits from the biological nitrogen fixation (BNF) carried out within infected cells of the central nodule tissue by differentiated rhizobia referred to as bacteroids (Brewin 1991; Cohn et al.

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The *SucS* sequences and the MtGI TC or GenBank accession numbers are as follows. *M. truncatula*: *MtSucS1* (full-length cDNA AJ131964), *MtSucS2* (599 amino acids encoded by the incomplete TC62069), *MtSucS3* (full-length TC67958), *MtSucS4* (158 aa encoded by the incomplete TC73525), and *MtSucS5* (374 aa encoded by the incomplete TC65865). *P. sativum*: *PsSus1* (full-length cDNA AF079851), *PsSus2* (full-length cDNA AJ001071), and *PsSus3* (full-length cDNA AJ311496).

1998). One key metabolic role of SucS in root nodules is to provide hexoses that can be metabolized to generate carbon skeletons necessary for the incorporation of ammonia released from nitrogen-fixing microsymbionts. Due to its abundance in root nodules, SucS was the first enzyme to be identified as being nodule-enhanced (Morell and Copeland 1985). In the meantime, Gordon and associates (1999) provided experimental evidence that SucS activity is essential for symbiotic nitrogen fixation.

Whereas the nodulation process is restricted to legumes, the formation of an arbuscular mycorrhiza (AM) with fungi of the order Glomales is widespread among terrestrial plants (Brundrett 2002). In AM, an extensive external fungal mycelium is associated with the colonized host root and facilitates bidirectional nutrient transfer. In return for carbohydrates that are supplied to the fungus (Shachar-Hill et al. 1995), the micro-symbiont translocates nutrients to the host, in particular phosphate absorbed from the soil (Smith et al. 2001). The interface of host nutrient uptake is assumed to be located predominantly within specific inner cortical cells that are invaginated by intraradical fungal hyphae. Subsequently, these develop into highly branched symbiotic structures designated arbuscules (Bonfante and Perotto 1995; Holley and Peterson 1979). While SucS are known to be crucial for carbon supply during BNF, its relevance during mycorrhization remains to be demonstrated. As a prerequisite, the poorly understood mechanisms of carbohy-

drate supply to the fungus during mycorrhization (Bago et al. 2000) have to be elucidated.

The application of the Mediterranean legume *M. truncatula* Gaertn. (barrel medic) as a model plant (Barker et al. 1990) opens the possibility to investigate both the nodule and the AM endosymbiosis in a legume that can be efficiently transformed and regenerated. We previously isolated the *MtSucS1* gene encoding a nodule-enhanced SucS (Hohnjec et al. 1999). The aim of this study was to generate transgenic *M. truncatula* plants harboring fusions of the *MtSucS1* promoter to the *gusAint* reporter gene to deduce the *MtSucS1* expression profile in endosymbiotic tissues, in order to assess the involvement of *MtSucS1* in these interactions. In this report, we demonstrate that *MtSucS1* is the only *M. truncatula* SucS gene known so far that is specifically up-regulated in root endosymbioses. On the cellular level, we provide evidence that *MtSucS1* is highly expressed in the infected region of root nodules as well as in arbuscule-containing and surrounding cortical cells of mycorrhizal roots, both in the model legume *M. truncatula* and in the grain legume crop *P. sativum*. The observed expression regulation implies that *MtSucS1* is involved in generating sink-strength, not only in root nodules but also in mycorrhizal roots.

RESULTS

Construction of transgenic *Medicago truncatula* plants expressing fusions of different regions of the *MtSucS1* gene to the reporter gene *gusAint*.

Based on the previously isolated *MtSucS1* gene (Hohnjec et al. 1999), we constructed two fusions of different promoter and gene fragments to the *gusAint* reporter gene (Vancanneyt et al. 1990) to characterize their activity in different organs and, in particular, during *M. truncatula* root endosymbioses. The first construct (Fig. 1) ranged from position –2050 to +800, relative to the transcriptional start site of the *MtSucS1* gene. In addition, the first exon (47 bp), the leader intron I (747 bp), and 6 bp of exon II were included, to allow a correct splicing of intron I. We decided to use this extended promoter to ensure a maximum potential activity, since both the exon I and the leader intron I of *SucS* genes stimulate gene expression in other plants. To prove whether the 3' region of *MtSucS1* is able to influence promoter activity, as shown for a *SucS* gene from potato, the CaMV 35S terminator (Gardner et al. 1981) present in construct –2050/+800 was replaced by the 3' region of the *MtSucS1* gene starting from the stop codon (Fig. 1). In this second construct, designated –2050/+800/3', the *gusAint* gene was terminated by the native *MtSucS1* 3' region of 1887 bp. Both the –2050/+800 and the –2050/+800/3' constructs contain a sequence from position –596 to –578 (Fig. 1) that shows homologies to a SURE (sucrose-responsive) element of the potato class I *patatin* promoter (Grierson et al. 1994), which is activated by the sucrose-inducible nuclear protein SURF (sucrose-responsive factor).

Fig. 1. Schematic representation of the –2050/+800 and –2050/+800/3' regions of the *MtSucS1* gene, which control *gusAint* reporter gene expression in transgenic tissues. **A**, Numbers indicate the position relative to the transcriptional start site of the *MtSucS1* gene. Boxes shaded in dark grey represent exon sequences (set in brackets when partial), while intron sequences are colored in light grey. The *gusAint* gene and the CaMV 35S terminator are indicated by a black arrow and a white box, respectively. The promoter and the nontranscribed native 3' region are shown as black bars. The position of a motif similar to the sucrose-responsive (SURE) element of the potato *patatin21* promoter is indicated by a triangle. **B**, Comparison of the two SURE motifs (boxed) of the potato SURE element present in the *patatin21* promoter with a region of the *MtSucS1* gene. Identical nucleotides are shown in bold type.

Fig. 2. Histochemical localization of GUS activity in *Medicago truncatula* plants transformed with the –2050/+800 *gusAint* fusion. GUS assays of the different organs were performed. The trifoliolate leaves as well as the whole flowers, pods, and seeds were photographed either in bright- or dark-field with a binocular, whereas semithin sectioned (80 to 120 µm thick) leaves, stems, pods, and roots were photographed in dark-field microscopy. **A**, Trifoliolate leaf showing GUS activity in the vein network. **B**, Cross section of a leaf showing GUS activity in the veins. **C**, Transversal stem section exhibiting intense staining in the phloem tissue and the cambium cells in addition to weak staining located in the chlorenchyma tissue and the xylem parenchyma. **D**, GUS staining in the flower. **E**, Magnification of the flower tissue with staining in the filament, anthers, theca, and pollen grains. **F**, Overall GUS staining pattern of a non-mature pod. **G**, Cross section through a young pod with GUS staining in the vasculature, the nutritive tissue surrounding the developing embryo, and the parenchyma cells. **H**, Cross section of a mature pod with weak GUS staining restricted to vascular bundles. **I**, GUS activity in the seed coat of young seeds. **J**, No GUS activity is detected in the seed coat of mature seeds. **K**, No GUS activity is detected in the mature cotyledons and the radicle. **L**, GUS activity of swollen mature seeds appears in the basal parts of the cotyledons and the radicle. **M**, Seedlings germinated for 48 h, showing intense staining in the root and in particular the root tip. **N**, GUS staining in mature roots. **O**, Root transverse and **P**, root longitudinal sections showing GUS activity in the central vascular cylinder, in particular the phloem vessels and phloem parenchyma. **Q**, GUS activity in a lateral root with a growing root apex.

The *gusAint* fusions were introduced into *M. truncatula* R108-1 via *Agrobacterium tumefaciens*-mediated transfer. In total, 30 transgenic plants of the T₀ generation were regenerated by somatic embryogenesis following leaf-disk transformation (Hoffmann et al. 1997; Trinh et al. 1998). In 24 T₀ lines, the *gusAint* gene was preceded by the -2050/+800 region, whereas six T₀ lines carried the *gusAint* gene under control of the -2050/+800/3' construct. A stable inheritance of the transgenes was verified by histochemical GUS assays, polymerase chain reaction (PCR) amplifications of the *gusAint* gene from genomic DNA, and by Southern blot hybridizations using the *gusAint* gene as a probe (data not shown).

The *MtSucS1* promoter extended by exon I and intron I directs gene expression to different sink tissues.

From selected stable lines of the T₂ and T₃ descendants that showed detectable GUS-staining of comparable strength in leaves, we collected several organs from different individual plants at about the same developmental stage for a comparative analysis of *MtSucS1* promoter activity (Fig. 2). In general, the *MtSucS1* promoter extended by exon I- and intron I-mediated expression in all vascular tissues examined, namely in leaves (Fig. 2A and B), stems (Fig. 2C), petals (Fig. 2D), anthers (Fig. 2D and E), pods (Fig. 2F, G, and H), seedlings (Fig. 2M), and roots (Fig. 2M through Q).

GUS activity in mature leaves was exclusively confined to the vein network (Fig. 2A and B). When investigating transversal stem sections, strongest expression was observed in phloem tissues and, to a lesser extent, in the chlorenchyma and the xylem parenchyma but was absent in phloem fibers and in the angular chlorenchyma (Fig. 2C). GUS-staining in flowers occurred in the theca and the pollen grains in addition to the vasculature of filaments and anthers (Fig. 2D and E). Green pods of mature size showed an overall reporter gene expression that was particularly strong in the spikes (Fig. 2F).

During seed development and maturation, the pattern of GUS activity exhibited a shift. In transversal sections through young pods, the promoter directed strongest expression to the pod vasculature as well as to the nutritive tissue surrounding the developing embryo, including the embedded vascular bundles (Fig. 2G). By contrast, weak GUS-staining in transversal sections of mature pods was restricted to the vasculature (Fig. 2H).

An examination of young and immature seeds revealed GUS activity in the testa (Fig. 2I) that was no longer present at later developmental stages (Fig. 2J). In mature embryos, promoter activity was absent (Fig. 2K) but, shortly after swelling in the light for 4 h, the promoter regained activity in the basal region of the cotyledons as well as in the radicle (Fig. 2L). This staining focused on the endosperm and the epidermal cell layers. Further germination for 48 h in the dark lead to activation in the root vasculature, the root tip, and, to a lesser extent, in the cortical root cells, as well as the tips of the cotyledons (Fig. 2M). Root-tip staining was still observable in mature root systems (Fig. 2N). An examination of semithin transversal (Fig. 2O) and longitudinal (Fig. 2P) sections of roots from adult plants revealed that the promoter fusion directed strongest *gus* expression to the central cylinder. Here, it was restricted to the phloem tissue including the parenchyma. Only in regions in which lateral roots just emerged from the cortex was *gus* expression present in the cortical cells of the main root and the meristematic region of young root tips (Fig. 2Q).

Considering that some plants generate endogenous GUS-like activity in different tissues (Hänsch et al. 1995; Hu et al. 1990) and since Journet and associates (2001) reported on the presence of background staining in *M. truncatula* cv. Jemalong, we assayed nontransformed *M. truncatula* R108-1 plants. Here,

background staining was only detectable at a very low level in the testa, the root vasculature, the radicle meristem of swollen seeds, in seeds germinated for 48 h, and in the anthers and pollen grains but was in no way comparable to that of the GUS-staining displayed by the transgenic plants (data not shown).

All expression patterns presented above were also obtained with the -2050/+800/3' reporter gene fusion (data not shown). Hence, no significant effect of the presence of the native *MtSucS1* 3' region on the localization of reporter gene activity was observed. The whole-plant expression pattern of the *MtSucS1* gene is in accordance with a function of the encoded SucS enzyme in sink tissues.

The -2050/+800 region of the *MtSucS1* gene mediates strong activity in the nodule cortex and in the infected cells of the central nodule tissue.

In this study, our main interest focused on the expression properties of *MtSucS1* during root endosymbioses, since root nodules and roots infected by AM fungi represent specialized symbiotic sink tissues.

A strong induction of *MtSucS1* in root nodules of *M. truncatula* was already shown on the transcript and protein level (Hohnjec et al. 1999). To investigate the cellular localization of *MtSucS1* promoter activity during symbiotic nitrogen fixation in detail, we nodulated transgenic roots expressing the -2050/+800 reporter gene fusion using the microsymbiont *Sinorhizobium meliloti* 1021 (Meade and Signer 1977). Longitudinal sections of mature nodules 21 to 25 days postinoculation (dpi) were assayed for *gus* expression (Fig. 3A through E). In the nodule periphery, strong GUS-staining was located in the inner cortex that envelops the nodule interior (Fig. 3A and B) and the nodule vascular bundles (Fig. 3A). In the central tissue, strong expression occurred in the apical region corresponding to the meristem and the prefixing zone II (Fig. 3D). Following the establishment of a nitrogen-fixing symbiosis, we detected less GUS activity in parts of the interzone II-III but an increased activity in cells of the nitrogen-fixing zone III (Fig. 3D) that slightly decreased towards the senescence zone IV (Fig. 3C). A closer examination of the nitrogen-fixing zone III revealed that the -2050/+800 region directed strongest *gus* expression to infected cells (Fig. 3E, black arrow), whereas uninfected cells showed either no or a weaker staining (Fig. 3E, white arrow).

The *MtSucS1* gene is induced in arbuscule-containing and surrounding cortical cells in response to colonization by *Glomus mosseae*.

With respect to gene induction prior to and during endosymbiosis, AM colonization shares common features with rhizobial interactions (Hirsch and Kapulnik 1998). In the case of *MtSucS1*, searches of the TIGR *M. truncatula* Gene Index (MtGI) (Quackenbush et al. 2000) revealed that, compared with uninfected roots, *MtSucS1* transcripts belonging to the tentative consensus sequence (TC) 67957 are of higher abundance, not only in nodulated but also in mycorrhizal roots of *M. truncatula*. Hence, we evaluated the cellular localization of *MtSucS1* promoter activity in response to mycorrhizal colonization by studying *G. mosseae*-inoculated roots of transgenic plants carrying the -2050/+800 *gusAint* fusion. After 25 days, the expression pattern from fungus-infected root sections (Fig. 3F and G) significantly differed from that observed in uninfected roots and described above (Fig. 2O and P). In contrast to noncolonized root segments (Fig. 3F and G, red arrow), we found *gus* expression in those cortical cells that surround the region in which arbuscule formation occurs (Fig. 3F and G, black arrow). Semithin sections of a highly colonized region identified intensely stained single cells in the innermost cortical

cal cell layer close to the central cylinder. These were highlighted against a background of cortical cells that exhibited an evenly distributed staining at a lower level (Fig. 3H and I). A more detailed localization of *MtSUC1* expression in this area revealed that cortical cells exhibiting strongest *gus* expression harbored arbuscules (Fig. 3J and K), while the surrounding noncolonized cortical cells were constantly stained weaker, irrespective of a direct contact to fungal hyphae or arbuscule-containing cells. Arbuscule-dependent staining was not only confined to cells with a completely developed structure but also occurred in cells that might contain young arbuscules (Fig. 3K, white arrow). Counterstaining with Chlorazole-black-E proved that the activity of the -2050/+800 region and the formation of arbuscules were located in the same cortical cells (data not shown).

Interestingly, even the internal and external hyphae exhibited a typical blue GUS coloration, as already mentioned by Journet and associates (2001). Since the staining was restricted to compartments (Fig. 3J, K, and N), the hydrophobic GUS complex might be stored within the lipid bodies recently described by Bago and associates (2002b). Consequently, this pattern might visualize the proposed frequent flow of lipids in the vicinity of fungal carbon uptake (Bago et al. 2002a and b). In control experiments, mycorrhizal roots of nontransgenic plants did not display any GUS-staining (data not shown), excluding the possibility that the blue coloration in the hyphae was due to fungal GUS activity.

In root nodules and endomycorrhizal roots, no qualitative differences in GUS activity were evoked by the -2050/+800/3' fusion (data not shown).

***MtSUC1* is the only SucS gene strongly induced during endosymbiotic interactions with rhizobia and arbuscular mycorrhizal fungi.**

In the TIGR *M. truncatula* Gene Index, apart from the *MtSUC1* gene represented by TC67957, expressed sequence tag-clustering identified four additional *MtSUC* genes. Two of them (TC62069 and TC67958) were referred to as *MtSUC2* and *MtSUC3*, respectively, based on their homologies to the *PsSUS2* and *PsSUS3* genes from *P. sativum* (Table 1), whereas TC73525 and TC65865 were designated *MtSUC4* and *MtSUC5*. To determine whether, apart from *MtSUC1*, any of the other four *MtSUC* genes were significantly induced in different *M. truncatula* tissues, we investigated their expression profile by quantitative real-time reverse transcription (RT)-PCR. The parallel amplification of transcripts encoding a constitutive translation elongation factor 1- α (*MtTef1*) (Wulf et al. 2003) served for normalization of gene expression (Fig. 4).

In nonsymbiotic tissues, the lowest accumulation of *MtSUC1* transcripts relative to *MtTef1* was observed in mature leaves, followed by young leaves. In relation to mature leaves, the expression of *MtSUC1* was enhanced 3.2-fold in flowers, 4.3-fold in seedlings, and 6.5-fold in young pods, but the highest induction was found in stems (15-fold) and uninfected roots (22-fold). These results confirm that, whenever strong *gus* expression was detected in transgenic plants, it correlates with enhanced abundance of *MtSUC1* transcripts.

Apart from *MtSUC1*, the genes *MtSUC2* and *MtSUC3* were also markedly expressed in diverse organs of the plant but exhibited their highest induction in other tissues than *MtSUC1*. *MtSUC2* was present in all tissues tested with the lowest transcript abundance in young pods, whereas the highest induction was measured in seedlings (sixfold) and stems (fourfold). Transcription of *MtSUC3* was lowest in mature leaves, while strongest abundance occurred in flowers (40-fold), followed by young developing pods (23-fold) and stems (16-fold). Unlike the *MtSUC1*, *MtSUC2*, and *MtSUC3* genes that were signifi-

cantly up-regulated in specific tissues, we detected no notable difference in RNA accumulation for both *MtSUC4* and *MtSUC5* in any of the organs analyzed (Fig. 4).

To investigate if, apart from *MtSUC1*, other *SucS* genes were up-regulated in root endosymbioses, we determined the relative transcript abundance of all five *MtSUC* genes in root nodules and AM. As a control, transcripts of the early nodulin gene *ENOD18* (Hohnjec et al. 2000) were amplified as a marker for nodulation, whereas detection of transcripts of the *M. truncatula* phosphate transporter *MtPT4* (Harrison et al. 2002) should prove an efficient mycorrhization. These experiments demonstrated an exclusive induction of *ENOD18* in nodules, whereas *MtPT4* expression was confined to mycorrhizal roots (Fig. 5).

Analyzing the *MtSUC1* transcript abundance in the tissues selected, we detected the highest induction in root nodules followed by mycorrhizal roots (Fig. 5). In relation to uninfected roots grown on fully supplemented medium, the transcript level of *MtSUC1* was 4.5-fold enhanced in nodules and 3.3-fold in AM roots. By contrast, a 2.5-fold enhanced expression occurred in mycorrhizal roots in relation to noninfected roots grown under similar conditions of phosphate limitation. No other *MtSUC* gene showed a significant up-regulation in symbiotic tissues, but *MtSUC2* and *MtSUC3* were induced 20-fold and 9.8-fold, respectively, in phosphate-limited control roots. Under phosphate depletion, plants respond with multiple morphological and physiological changes, e.g., enhanced root growth, induction of phosphate transporters, and an energy-mediated cotransport, in order to enhance phosphate acquisition (Raghothama 1999). Thus, both *MtSUC2* and *MtSUC3* might support the alterations in metabolism necessary to overcome phosphate deficiency.

The *MtSUC* gene family encodes orthologues of the three SucS isoforms identified in the grain legume *P. sativum*.

To define orthologous *SucS* genes in the model legume *M. truncatula* and the grain legume *P. sativum*, we aligned the deduced amino acid sequences of both species, using Dialign 2.2 (Morgenstern et al. 1999). For this approach, we chose the full-length cDNA sequences of *MtSUC1* (Hohnjec et al. 1999) and *MtSUC3* (TC67958), together with the incomplete tentative consensus sequences of *MtSUC2* (TC62069), *MtSUC4* (TC73525), and *MtSUC5* (TC65865). In *P. sativum*, four full-length *SucS* cDNAs were published so far, with two of them encoding the *PsSUS1* gene with an identity of 99.5% to each other on the level of proteins (nodule-enhanced *SucS*, GenBank accession AF079851; *rug4* gene, Craig et al. 1999). Table 1 summarizes the identities of the five *MtSUC* and the three *PsSUS* sequences. In this alignment, the nodule-enhanced *MtSUC1* exhibited the highest homology to *PsSUS1* (97%). The *MtSUC2* sequence shared the best identity (95.8%) to *PsSUS2* (Buchner et al. 1998), whereas *MtSUC3* was 93.3% identical to *PsSUS3* (Barratt et al. 2001). *MtSUC4* and *MtSUC5* showed lower identities to the three *SucS*s of *P. sativum* and could correspond to two additional *PsSUS* genes identified by Southern blotting (Barratt et al. 2001). Based on the alignment, a phylogenetic tree was constructed that reflects the relationship between all *SucS* enzymes. Here, *SucS* isoforms 1, 2, and 3 from both plant species grouped together on distinct branches of the tree, while *MtSUC4* and *MtSUC5* ranked at a more distant position (Fig. 6). It should be noted also that the expression of *MtSUC1*, *MtSUC2*, and *MtSUC3* is similar to that of the three corresponding *SucS* genes of *P. sativum* (Barratt et al. 2001). Here, *PsSUS1* was expressed in all plant organs examined, whereas *PsSUS2* was detected in the old testa as well as in young and mature leaves. As observed for *MtSUC3*, the *PsSUS3* gene showed its maximal induction in

Fig. 3. Histochemical localization of GUS activity in endosymbiotic tissues of root nodules and mycorrhized roots transformed with the -2050/+800 *gusAint* fusion. **A** through **E**, GUS activity in nodules of transgenic *Medicago truncatula* plants 21 to 25 days postinoculation. Prior to dark-field microscopy, nodules were semithin sectioned at 60 to 80 μ m. **A**, Magnification of the peripheral region of a root nodule showing strong staining in the inner cortical cells and the nodule vascular bundle. **B**, Magnification of the peripheral nodule region with a continuous staining of noninfected cells adjacent to the inner cortex. **C**, Overview of GUS activity in a complete root nodule section. **D**, Magnification of the nodule apex, in which strongest GUS staining is observed in the meristematic cells and the adjacent prefixing zone II. **E**, Magnification of the nitrogen-fixing zone III, in which strong activity occurs in the infected cells (black arrowhead), whereas only weak staining is detected in uninfected cells (white arrowhead). **F** through **K**, GUS activity in mycorrhized roots of transgenic *M. truncatula* plants performed 25 days after inoculation with *Glomus mosseae*. **F**, Root with both intensively colonized (black arrowhead) and noncolonized (red arrowhead) segments photographed in bright-field. **G**, Regions of a root mycorrhized to a lower extent (black arrowhead) compared to noncolonized root parts (red arrowhead) photographed in dark-field. **H**, Semithin section (100 μ m) of a root fragment with GUS activity in parenchyma cells and strongest staining in the cortex cell layer close to the vasculature. **I**, Magnification of the innermost cortical cell layer bearing arbuscules. **J** and **K**, Strong GUS staining located in arbuscule-containing cells. The GUS precipitate outlines the contour of the arbuscules in addition to the internal hypha and is present in both small and large phases of arbuscules (K, left hand site). **L** through **N**, GUS activity in transgenic nodules and transgenic mycorrhized roots of *Pisum sativum* 21 to 25 days postinoculation with the appropriate microsymbionts. **L**, Semithin (60 to 80 μ m) nodule section photographed using bright-field microscopy. **M**, GUS activity in a semithin section (100 μ m) of a highly colonized root segment, in which intensely stained cells are located in cortical cells nearby the vasculature, surrounded by weaker staining throughout the parenchyma cells. **N**, Magnification of an arbuscule-containing cell showing strong GUS activity in cortical cells surrounding the arbuscules.

Table 1. Homologies of *Medicago truncatula* and *Pisum sativum* sucrose synthase (SucS) sequences expressed in percentage of identical amino acids

Sequence	MtSucS1	MtSucS2	MtSucS3	MtSucS4	MtSucS5
PsSus1	97.0	74.7	88.5	63.4	65.5
PsSus2	70.4	95.8	69.5	87.7	67.6
PsSus3	86.7	74.3	93.3	61.9	67.4

Fig. 4. Relative induction of five *MtSucS* genes in different organs of *Medicago truncatula* revealed by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). *SucS* inductions were normalized against *MtTef* gene expression, which was amplified as a constitutive control. Samples from plant organs were obtained by pooling tissues from 10 different plants. Three independent RT-PCR experiments were performed for each gene. The arithmetic mean and the standard deviation of these data are presented here.

flowers. It is thus evident that *MtSucS1*, *MtSucS2*, and *MtSucS3* represent orthologues of the three pea *SucS* genes isolated so far.

The -2050/+800 region of *MtSucS1* is active in endosymbiotic tissues of pea.

The existence of an *MtSucS1* orthologous in pea tempted us to test whether the expression pattern mediated by the -2050/+800 region in *M. truncatula* endosymbioses was also observed in the heterologous system *P. sativum*. We thus generated pea hairy roots that expressed the -2050/+800 reporter gene fusion. These hairy roots were either nodulated with *Rhizobium leguminosarum* VF39 (Priefer 1989) or inoculated with the arbuscular mycorrhiza fungus *G. intraradices* and were subsequently assayed for *gus* expression after 21 to 25 dpi.

In transgenic pea nodules, the peripheral tissues, the inner cortex, and the vascular bundles appeared intensely stained.

Fig. 5. Relative induction of five *MtSucS* genes in sterile roots, nodules, nonmycorrhized (myc⁻) roots, and mycorrhized (myc⁺) roots of *Medicago truncatula*. Using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), the induction of five *MtSucS* genes as well as the nodule-specific *MtENOD18* and the mycorrhiza-induced *MtPT4* genes were normalized against the constitutive control *MtTef*. Samples from plant organs were obtained by pooling tissues from 10 different plants. Three independent RT-PCR experiments were performed for each gene. The arithmetic mean and the standard deviation of these data are presented here.

Concerning the central tissue, the highest expression concentrated in the nodule apex corresponding to the meristem and the prefixing zone II, followed by a slight decrease in the interzone II-III and a strong GUS activity mainly restricted to the infected cells of the nitrogen-fixing zone III (Fig. 3L). In contrast to in situ hybridizations performed by van Ghelue and associates (1996), the uninfected cells of the central nodule tissue were stained only very weakly. This might be due to the use of a heterologous system for promoter analysis. In highly mycorrhized root segments, *gus* expression occurred both in root cortical cells and arbuscule-containing cells with the strongest induction in cells harboring fungal structures (Fig. 3M and N). Our results indicate that regulatory elements mediating expression in symbiotic sink organs are largely conserved between the model legume *M. truncatula* and the grain legume *P. sativum*.

DISCUSSION

The *MtSUC1* gene is induced in phloem and sink tissues.

Our observation of *MtSUC1* promoter activity in the entire phloem tissue of leaves, stems, and roots corresponds to studies on the *A. thaliana AtSUC1* (Martin et al. 1993) and the maize *Shrunken1* gene (Yang and Russell 1990). In leaf and stem veins, a general induction of *MtSUC1* is consistent with a role of the encoded enzyme in the process of apoplastic sucrose loading into companion cells and sieve elements (Ward et al. 1998, Patrick et al. 2001). Phloem loading against a sucrose concentration gradient is supposed to involve sucrose/H⁺ cotransporters (SUT) energized by a plasma membrane H⁺-ATPase (Riesmeier et al. 1992). The ATP supply to maintain this mechanism is thought to depend on the cleavage of sucrose by SucS (Martin et al. 1993). In addition, assimilates that leaked to the phloem apoplast during long-distance transport in stems are probably retrieved into the sieve element companion cell complexes by the same active import (Patrick et al. 2001). Within roots, *MtSUC1* promoter activity focuses on the primary phloem, in which SucS may provide hexoses for cell wall biosynthesis and deposition (Hendrix 1990). Furthermore, *MtSUC1*-mediated expression in the central cylinder of roots and in the vasculature of other organs might be associated with the unloading process and post-phloem transport that is meant to occur predominantly by symplastic movement. In this context, the SucS activity is discussed to determine the quantity of sucrose allocation from source to sink (Martin et al. 1993; Nguyen-Quoc and Foyer 2001; Sturm and Tang 1999). Accordingly, the change from strong induction of *MtSUC1* in the vasculature and the nutritive tissue of young pods to reduced activity in mature pods might reflect a change in sink demand at a stage when the need for hexoses to support seed maturation and storage processes decreased. It is known that patterns of *SucS* expression shift subjected to the physiological or developmental stage of an organ. Thus, a stringent regulation with respect to the state of sink strength is plausible (Bush 1999). The *MtSUC1* promoter was also found to direct *gus* expression to meristematic regions of the tips of germinating

radicles, seedling roots, and growing lateral roots. A similar pattern of expression was observed, e.g., for *Sus3* in potato roots (Fu and Park 1995). The up-regulation in cell division zones of roots suggests that *MtSUC1* provides substrates for the high metabolic turnover required to support cell division, elongation, and differentiation. Activation of *MtSUC1* was also detected in the theca and the pollen grains at a late stage of flower development when anthers and pollen grains were already differentiated. Therefore, in addition to invertases (Ylstra et al. 1998), *MtSUC1* may exhibit specific functions in providing hexoses for the active metabolism of this reproductive organ, e.g., during pollen maturation and pollen tube growth (Datta et al. 2001 and 2002).

The *MtSUC1* expression pattern in root nodules indicates a role for *MtSUC1* in generating the sink-strength of this symbiotic tissue.

A 4.5-fold induction of *MtSUC1* in nodules as determined by RT-PCR is comparable to results obtained in *Vicia faba* and *M. truncatula*, in which an up to 10-fold induction was found by Northern hybridizations (Küster et al. 1993; Hohnjec et al. 1999). On the cellular level, our histological assays in transgenic legume nodules pointed to an induction of *MtSUC1* in the nodule cortex, in the meristematic region, and in the central nodule tissue. Here, *MtSUC1* activity was strongest in infected and only weak in uninfected cells. This conforms to transcript and enzyme localizations in root nodules of *Medicago sativa* and *Phaseolus vulgaris* (Trepp et al. 1999; van Ghelue et al. 1996).

The nodule cortex is involved in assimilate transport from the vasculature to the central tissues (Morell and Copeland 1985). Since we observed strong *MtSUC1* promoter activity in cortical cells, it can be assumed that, apart from sucrose, hexoses also are translocated to the inner region of root nodules. Based on studies in *V. faba*, Abd-Alla and associates (2000) described a symplastic link from the vascular bundles to the infected region through an enhanced presence of plasmodesmata, whereas symplastic connections between infected cells were found to be nearly absent. Thus, the authors proposed that infected cells receive their metabolites mainly via the noninfected cells. This corresponds to previous conclusions for determinate soybean nodules (Gordon et al. 1992). Unlike *Glycine max*, in which a predominant breakdown of sucrose was suggested to be confined to the noninfected cells (Gordon et al. 1992), the expression pattern of *MtSUC1* clearly favors an allocation of sucrose also to infected cells, with a subsequent cleavage therein. An elevated demand of hexoses in infected cells was described to support enhanced respiration, the generation of carbon compounds for incorporation of ammonia, the production of carbon sources for the nitrogen-fixing bacteroids, and the biosynthesis of cellulose (Thummler and Verma 1987). Apart from this, sucrose degradation could support the ATP production necessary for the H⁺-ATPases that are located in the peri-bacteroid membrane and acidify the peri-bacteroid space (PBS). The shift in pH value in this interface for nutrient exchange is prerequisite to a formation of ammonia in the PBS and its subsequent import into host cells via ammonia channels (Day et al. 2001).

Our data indicate that, out of five SucS genes analyzed, only *MtSUC1* is up-regulated in root nodules, thus pointing to a crucial role of the encoded enzyme during BNF. This conforms to studies on the *rug4* mutant (Wang and Hedley 1993) of pea, which is highly reduced in its PsSus1 activity, leading to a phenotype of wrinkled seeds and to a strongly impaired development when being dependent on symbiotic nitrogen fixation (Craig et al. 1999). Detailed physiological analyses clearly demonstrated that the *MtSUC1* orthologue PsSus1 was essen-

Fig. 6. Phylogenetic relationship of *Medicago truncatula* and *Pisum sativum* sucrose synthases (SucS). Based on SucS sequences from *M. truncatula* and pea currently available, a multiple sequence alignment was performed using Dialign 2.2, and a phylogenetic tree was constructed using Indiana University's Phylodendron software version 0.8d. The lengths of the branches reflect the sequence diversity counted as the number of substitutions per residue.

tial for an effective nitrogen fixation and, despite their presence in root nodules, alkaline invertases were not able to compensate for this (Gordon et al. 1999). Thus, the pure ability to cleave sucrose is not sufficient to explain why *MtSUC1* orthologues act as limiting factors for the entire nodule metabolism. A stringent metabolic control by SucS under several stress conditions was already demonstrated in different species and has been discussed to govern the rate of carbon entry into the respiratory path, thus allowing the plant to adjust to altered sugar supply (Arrese-Igor et al. 1999; Koch et al. 1992). Analogous to pea, the complex expression regulation of *MtSUC1* in nodules opens the possibility to control nitrogen fixation through *SucS* expression and SucS activity, as proposed by Gordon and associates (1997).

The *MtSUC1* expression pattern in mycorrhizal roots indicates that *MtSUC1* supports the metabolism of both the arbuscule-containing and the surrounding cortical cells.

In root segments extensively colonized with AM fungi, the *MtSUC1* promoter, in addition to the vasculature, was predominantly active in cells containing arbuscules and in the surrounding noncolonized cortical cells. Since this up-regulation was observed as a response to the presence of a fungal micro-symbiont, the encoded SucS might support at least a part of the elevated hexose demand induced by the micro-symbiont in colonized regions of the root (Shachar-Hill et al. 1995). Moreover, the *MtSUC1* expression pattern supports the hypothesis that, apart from arbuscule-containing cells (Blee and Anderson 1998), the intraradical hyphae might represent the predominant interface for hexose supply to the fungus (Bago et al. 2002a; Harrison 1999). The assumed involvement of *MtSUC1* in the generation of sink strength conforms not only to Graham (2000), who reported an up to 20% increase in assimilate allocation to a mycorrhizal root system, but also to the numerous evidences for highly elevated metabolic activity and respiration in mycorrhizal associations (Gianinazzi-Pearson 1996; Holley and Peterson 1979).

In addition to cortical cells, the *MtSUC1* promoter displayed strongest activity in cells harboring arbuscules. Here, sucrose cleavage by SucS could additionally be essential for the formation of an ATP-dependent proton gradient across the periarbuscular membrane (PAM). Proton-coupled uptake mechanisms are supported by the presence of plant H^+ -ATPases that are restricted to the PAM (Gianinazzi-Pearson et al. 1991), thus rendering the arbuscule-containing cells the assumed site of host nutrient uptake (Gianinazzi-Pearson 1996; Harrison 1999). In this context, sucrose breakdown might be particularly relevant to facilitate the phosphate acquisition across the interface, since plant phosphate transporters are thought to be energy-dependent $H^+/H_2PO_4^-$ cotransporters (Smith et al. 2003).

Interestingly, the characteristics of *MtSUC1* expression during endomycorrhiza share similarities to that of *Mtst1*. This *M. truncatula* gene encoding a hexose transporter is mainly expressed in the phloem of roots and in the elongation zone of root tips, but its activity increases significantly in cortical cells of highly mycorrhized root segments (Harrison 1996, 1997). This raises the possibility that, in the vicinity of fungal structures, a cytoplasmic sucrose breakdown by *MtSUC1* can be performed in the same cells in which hexoses are thought to be imported from the apoplast by *Mtst1*. It is feasible that the purpose of hexose import into cortical cells is that of controlling or fine-tuning the amount of hexoses allocated to the fungus (Harrison 1996; Smith et al. 2001), since an "uncontrolled" sink strength due to the rapid conversion of hexoses to trehalose and glycogen within the hyphae (Shachar-Hill et al. 1995) might lead to physiological deficits of the host. Hexose re-

uptake by cortical cells would not only recover a fraction of assimilates but might additionally contribute to a control of SucS activity to prevent the further cytoplasmic breakdown of sucrose. The down-regulation of *SucS* expression in response to high glucose concentrations has been described in many plant species (Koch 1996; Koch et al. 1992) and, with respect to this, it should be noted that membrane-bound sugar transporters were discussed as possible factors involved in sugar sensing (Smeekens 2000).

Although we could exclude the possibility that other *MtSUC* genes present so far were up-regulated in mycorrhizal roots of *M. truncatula*, apoplastic invertases are known to be induced during different biotrophic interactions, including endomycorrhiza (Bago et al. 2000). Transcripts of a soluble acid invertase in addition to *SucS* were recently shown to accumulate during mycorrhization of *P. vulgaris* roots. The authors concluded that both types of enzymes establish a strong sink activity in arbuscule-containing cells. Since fluorescent cells, which were discussed to mark ineffective arbuscules, were mainly selected for analysis, it was proposed that *SucS* expression in arbuscule-containing cells might be correlated with the degradation of arbuscules and a re-establishment of the original cell organization (Blee and Anderson 2002). In the present study, we focused mainly on extensively colonized root loci, in which the amount of intraradical hyphae and arbuscule-containing cells suggests both the formation and degradation of arbuscules to occur in parallel. We therefore propose that *MtSUC1* is, at least, not exclusively involved in restoring the original status of an invaded cortical cell but, as outlined above, in addition to invertases, provides metabolites and generates sink strength during the active phase of the mycorrhiza symbiosis. This would be in accordance with observations by Ravnskov and associates (2003), who reported an increase of *SucS* gene expression during earlier stages of mycorrhizal root formation in maize.

Does *MtSUC1* fulfil an essential function for the endomycorrhiza symbiosis?

Experimental evidence that the nodule symbiosis strictly depends on an active PsSus1 enzyme was derived from studying the *rug4* mutant of *P. sativum* (Craig et al. 1999; Gordon et al. 1999). This mutant fails to grow without exogenously supplied nitrogen, and neither root nodule invertases nor other SucS isoforms were able to compensate for this deficiency. It is thus possible that, although invertases are induced in endomycorrhizal roots, *MtSUC1* could play an important role also during mycorrhization, by affecting this endosymbiosis in the same way as it was reported for the orthologous PsSus1 concerning root nodules. However, taking into account that post-transcriptional and -translational modifications are important control mechanisms for SucS (Winter and Huber 2000), our expression data can only be a first step, and further studies using mutants or transgenic plants with reduced SucS activity will be required to resolve this question.

MATERIALS AND METHODS

Isolation of nucleic acids, recombinant DNA techniques, and hybridizations.

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Plasmid DNA was prepared from *Escherichia coli* XL1Blue grown o/n on Pennassay agar plates containing appropriate antibiotics, using the Plasmid Mini Kit (Qiagen). Cloning experiments were carried out in *E. coli* XL1Blue (Bullock et al. 1987), using pK18 (Pridmore 1987) or pBluescript (Stratagene, La Jolla, CA, U.S.A.) plasmids. All in vitro manipulations of DNA were carried out using standard protocols (Sambrook et al. 1989).

Quantitative real-time RT-PCR.

Quantitative real-time RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR kit (Qiagen). DNaseI-treated RNA preparations were checked for the absence of DNA contaminations by a control PCR using *MtSUC1* primers spanning an intron. In these experiments, PCR amplifications from the respective genomic region were performed in parallel as a positive control. For RT-PCR, 50 ng of total RNA were used in a total volume of 25 µl. Reactions were run on the Opticon real-time PCR cycler (MJ Research, Waltham, MA, U.S.A.), and quantification was performed with the Opticon Monitor analysis software version 1.05. Every reaction was set up in three replicates. The program used was as follows: reverse transcription (50°C for 30 min), polymerase activation (95°C for 15 min), amplification and quantification cycles repeated 44 times (94°C for 15 s, 45°C for 30 s, 72°C for 30 s, with a single fluorescence measurement), and melting curve (40 to 95°C with one fluorescence read every 1°C). A relative expression ratio was calculated as the ratio of normalized gene expression of the gene of interest against a constitutively expressed *M. truncatula* gene that encodes an elongation factor 1- α (*MtTefa*, TC67846, TIGR *M. truncatula* Gene Index). The following formula was used: relative expression ratio of the gene of interest is $2^{-\Delta CT}$ with $\Delta CT = CT_{\text{gene}} - \text{minus } CT_{\text{Tefa}}$. The gene-specific primers used for RT-PCR are listed in Table 2. All primers had a calculated T_m of $53 \pm 0.1^\circ\text{C}$, amplification products were not larger than 299 bp, and the primer sequences were unique in the TIGR *M. truncatula* Gene Index.

Construction of *Agrobacterium* sp. strains carrying binary vectors with fusions of different regions of the *MtSUC1* gene to the *gusAint* reporter gene.

A PCR fragment covering the -2050 to +800 region of the *MtSUC1* gene was amplified, using the previously isolated *MtSUC1* gene (Hohnjec et al. 1999) as a template. For cloning purposes, a restriction site for *HindIII* was introduced flanking the primer sequence from position +800 to +780. After sub-cloning using the native *EcoRI* site at position -2050 and the artificial *HindIII* site at position +800, the -2050 to +800 region was inserted as an *EcoRI/HindIII* fragment upstream of the *gusAint* reporter gene (Vancanneyt et al. 1990) on vector pGUSINT (Küster et al. 1995). The promoter region cloned and the junctions of the *gusAint* fusion were verified by sequencing. In a second construct designated -2050/+800/3', the CaMV 35S terminator present downstream the *gusAint* gene was replaced by a PCR-amplified 1,887-bp fragment (carrying flanking *KpnI* and *NotI* restriction sites introduced in the PCR primers) from the 3' region of the *MtSUC1* gene. Both *gusAint* fusions were subsequently cloned as *SmaI/NotI* fragments into the binary vector MF2 Δ , a deletion derivative of the binary vector MF2 provided by F. Frugier (CNRS Gif-sur-Yvette, France). The original plasmid MF2 carried both a p35S-*gusAint*-pAnos construct and an additional p35S-pAnos cas-

sette flanking a multiple cloning site. A *HindIII/XbaI* deletion removed the p35S-*gusAint* sequence from the MF2 vector and made it suitable for cloning fusions of the *MtSUC1* promoter to the *gusAint* reporter gene. Using standard protocols, binary vectors carrying the *gusAint* fusions were electroporated into the *A. rhizogenes* strain Arqual (Quandt et al. 1993) to induce hairy roots and into *A. tumefaciens* EHA105 (Hood et al. 1993) to start leaf-disk transformations.

Transformation of *M. truncatula* and generation of transgenic hairy roots on *P. sativum*.

M. truncatula R108-1 (Hoffmann et al. 1997) plants were transformed with *MtSUC1* promoter-*gusAint* constructs according to Hoffmann and associates (1997) and Trinh and associates (1998), using *A. tumefaciens* strain EHA105 (Hood et al. 1993). Transgenic plantlets were selected by kanamycin-resistance. Deviations to the protocol were the augmentin (Smith-Kline Beecham, Brussels, Belgium) concentration (up to 1,200 mg per liter was used in callus media) and the use of different agar supplements (Phytigel [Sigma-Aldrich, Taufkirchen, Germany] at 3 g per liter for callus media, Phytoagar [Duchefa, Haarlem, The Netherlands] at 8 g per liter for embryo-inducing media).

The generation of transgenic hairy roots on *P. sativum* L. cv. Finale (Engvild 1987) was performed based on a procedure described for *Vicia hirsuta* by Quandt and associates (1993). To adopt the method for *P. sativum*, seeds swollen and germinated for 48 h in the dark were grown in small pots (3-cm diameter) with vermiculite/perlite (1:1) substrate that was fertilized either with nitrogen-free nodulation buffer (Quandt et al. 1993) for subsequent nodulation or with half-strength Hoagland's solution (Arnon and Hoagland 1940) for subsequent mycorrhization. After two days, seedlings were infected at the epicotyls, using derivatives of *A. rhizogenes* ARqual (Quandt et al. 1993) grown overnight at 30°C in petri dishes on LB medium (Sambrook et al. 1989) containing 100 mg of kanamycin per liter. *A. rhizogenes* cultures were dissolved in 3 ml of PS buffer (7 g of Na₂PO₄, 5 g of NaCl, and 3 g of KH₂PO₄ per liter) prior to infection, according to Quandt and associates (1993). After nine days, the infected area was covered by humidified vermiculite. Approximately three weeks after infection, wild-type roots were removed prior to transfer of composite plants to larger pots (two plants per pot of 11-cm diameter). Subsequently, hairy roots were inoculated with appropriate microsymbionts.

Conditions of plant growth and microsymbiont inoculation.

For the whole-plant expression pattern of the *gusAint* gene, seeds from *M. truncatula* R108-1 (Hoffmann et al. 1997) wild type and seeds from transgenic plants were surface-sterilized, as described by Trieu and associates (2000), were spread on 0.8% water agar (agar-agar; Sigma, Taufkirchen, Germany) in inverted petri dishes, and germinated for 40 h. Subsequently,

Table 2. Primer sequences used for quantitative real-time RT-PCR

Gene	Identifier ^a	Primer A	Primer B
Sucrose synthase (SucS) <i>MtSUC1</i> ^b	TC67957	attcaactgttagtctcgat	gttgagaatgaagagcacata
SucS <i>MtSUC2</i>	TC62069	aacaaaaatctgatggaggt	tcttattgtctgctgtcattc
SucS <i>MtSUC3</i>	TC67958	attcaaaatctggattcactt	cattaaagaccatagggtattc
SucS <i>MtSUC4</i>	TC73525	ttggtgaaattatcaagctcg	gttctctttgaagctgagat
SucS <i>MtSUC5</i>	TC65865	gagaaaaatcaagattgacc	gaagaatcctccaacaattac
Early nodulin <i>MtENOD18</i>	TC69023	atcaactccaattctctgat	tccagtagtatccttttgat
Phosphate transporter <i>MtPT4</i> ^c	TC59741	aatttgataggattcttttgc	ttcacatcttctcagttcttg
Translation elongation factor 1- α	TC67846	actgtgcagtagtacttggtg	aagctaggaggtattgacaa

^a Tentative consensus sequence from the TIGR *Medicago truncatula* Gene Index.

^b Hohnjec et al. 1999.

^c Harrison et al. 2002.

wild-type plants and T₂ and T₃ descendants of the transgenic lines were grown in sterile soil/vermiculite/sand (1:1:1) substrate in the growth chamber under a photoperiod of 16 h at 22°C with a decrease to 18°C during the dark phase. The relative humidity was set to 70%, and the plants were supplemented with commercial fertilizers.

For the expression pattern of the *gusAint* gene in endosymbioses, wild-type and transgenic seeds of *M. truncatula* cv. R108-1 (Hoffmann et al. 1997) were sterilized, as described above. For nodulation experiments with *S. meliloti* 1021 (Meade and Signer 1977), germinated seedlings were raised in vermiculite/perlite (1:1) substrate and were inoculated with rhizobia grown in TY medium (Beringer 1974) to an optical density at 580 nm of 0.8 to 1.0. These plants were fertilized weekly with nitrogen-free nodulation buffer, according to Quandt and associates (1993). In case of mycorrhization with *G. mosseae*, seedlings were grown in pots with autoclaved clay granulate (Seramis, Masterfoods GmbH, Verden, Germany) and were inoculated to 1/10 of the substrate volume with an inoculum containing spores of the AM fungus *G. mosseae* (Granular AMF Inoculum *G. mosseae* BEG 12; Biorize R&D, Dijon, France). Mycorrhized plants were fertilized weekly with half-strength Hoagland's solution (Arnon and Hoagland 1940) containing 20 µM phosphates.

After removal of wild-type roots, transgenic hairy roots of *P. sativum* were either nodulated with the *R. leguminosarum* VF39 (Priefer 1989) or inoculated with *G. intraradices* (Premier Tech Biotechnologies, Rivière-de-Loup, Québec, Canada) and fertilized according to the protocol used for the *M. truncatula* plants.

For quantitative real-time RT-PCR with tissues derived from *M. truncatula* cv. Jemalong A17 (T. Huguet, INRA Toulouse, France), we used total RNA extracted from seedlings germinated for 48 h in the dark, stem tissue, young leaves approximately 1 cm in diameter, mature leaves approximately 3 to 3.5 cm in diameter, mature flowers, immature pods approximately 3 to 4 mm long, sterile roots grown on petri dishes containing nodulation buffer, according to Quandt and associates (1993), supplemented with 20 mM NH₄NO₃, root nodules harvested at 21 to 25 dpi and grown in petri dishes containing nitrogen-free nodulation buffer, according to Quandt and associates (1993), mycorrhized (myc⁺) roots harvested 21 to 25 dpi with *G. intraradices* (Premier Tech Biotechnologies), and nonmycorrhized (myc⁻) roots. Myc⁺ and myc⁻ roots were fertilized weekly with half-strength Hoagland's solution (Arnon and Hoagland 1940), containing 20 µM phosphate and grown in pods with sterilized clay granulate (Seramis; Masterfoods GmbH, Verden, Germany). All plants were grown in growth chambers under a photoperiod of 16 h at 22°C with a decrease to 18°C during the dark phase. The relative humidity was set to 70%.

Histochemical analysis of transgenic tissues.

Prior to histochemical staining for GUS activity, all tissues were preheated to 55°C for 1 h to prevent background coloration (Hänsch et al. 1995; Hu et al. 1990; Journet et al. 2001) and were subsequently assayed for *gus* expression, based on the protocol of Jefferson and associates (1987). The GUS substrate solution contained 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt) (Biosynth AG, Staad, Switzerland), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 100 mM Tris, pH 7.0, 50 mM sodium chloride, pH 7.0, and 0.1% Tween-20. Seeds, pods, stems, flower, and leaf tissues were first vacuum-infiltrated prior to incubation in the dark at 37°C for 1 to 24 h. To improve the contrast between stained and nonstained cells, the assayed tissues were briefly cleared with 3% (vol/vol) sodium hypochloride prior to microscopy. GUS assays of transgenic *M. truncatula*

plants and *P. sativum* hairy roots expressing *gusAint* fusions were performed 21 to 25 days after nodulation with *S. meliloti* 1021 or *R. leguminosarum* VF39 and 21 to 25 days after mycorrhization with *G. mosseae*.

For staining fungal structures, roots were first cleared with 10% (wt/vol) potassium hydroxide for 10 min at 90°C and were subsequently assayed using 0.03% (wt/vol) Chlorazole-black-E in a lactoglycerol solution (1:1:1 lactic acid, glycerol, and water) for 4 h at 90°C. Prior to observation, the roots were immersed in 50% (vol/vol) glycerol for several days.

Tissues of transgenic plants were assayed for *gus* expression at different stages of development. Before staining, the material was prepared either by hand sections with razor blades or by semithin sections of 60 to 120 µm performed with a Leica VT1000S vibratome (Leica, Wetzlar, Germany). Documentation was carried out by Olympus BH-2 light microscopy (Olympus, Hamburg, Germany) and using an OM-PT binocular (Olympus, Hamburg, Germany), respectively. Photographs were taken using a C-2000Z digital camera (Olympus).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Indiana University Bio-Archive Phylodendron software version 0.8d:
iubio.bio.indiana.edu/soft/molbio/java/apps/trees
The Institute for Genomic Research (TIGR) *Medicago truncatula* Gene
Index: www.tigr.org/tdb/mtgi