

MtSWEET11, a Nodule-Specific Sucrose Transporter of *Medicago truncatula*^{1[OPEN]}

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Optimization of nitrogen fixation by rhizobia in legumes is a key area of research for sustainable agriculture. Symbiotic nitrogen fixation (SNF) occurs in specialized organs called nodules and depends on a steady supply of carbon to both plant and bacterial cells. Here we report the functional characterization of a nodule-specific Suc transporter, MtSWEET11 from *Medicago truncatula*. MtSWEET11 belongs to a clade of plant SWEET proteins that are capable of transporting Suc and play critical roles in pathogen susceptibility. When expressed in mammalian cells, MtSWEET11 transported sucrose (Suc) but not glucose (Glc). The *MtSWEET11* gene was found to be expressed in infected root hair cells, and in the meristem, invasion zone, and vasculature of nodules. Expression of an MtSWEET11-GFP fusion protein in nodules resulted in green fluorescence associated with the plasma membrane of uninfected cells and infection thread and symbiosome membranes of infected cells. Two independent *Tnt1*-insertion *sweet11* mutants were uncompromised in SNF. Therefore, although MtSWEET11 appears to be involved in Suc distribution within nodules, it is not crucial for SNF, probably because other Suc transporters can fulfill its role(s).

Legumes can establish nitrogen-fixing symbioses with soil bacteria called rhizobia that provide reduced nitrogen, primarily ammonia, to the plant for growth. In return, the bacteria receive reduced carbon from plant photosynthesis, along with all other nutrients required for metabolism and growth (Udvardi and Poole, 2013). Legume-rhizobia symbioses are a primary entry point for nitrogen (N) into the terrestrial biological N-cycle, which makes them key components of natural and agricultural ecosystems (Peoples et al., 2009). Over the last three decades, considerable progress has been made in our understanding of how various solutes are translocated between symbiotic partners. Nevertheless, key transporters, including those involved in transport of sugars into nodule cells and between cellular compartments, remain largely unknown (Udvardi and Poole, 2013; Clarke et al., 2014; Benedito et al., 2010).

Suc transport from phloem cells to cells in sink organs can occur in two ways: apoplasmic flow via plasma membrane (PM)-located transporters and symplasmic flow via plasmodesmal connections (Patrick, 1997). Increased frequencies of plasmodesmata have been documented in nodules of legumes (Complainville et al., 2003) and the non-legume, *Datisca glomerata* (Schubert et al., 2011). In nodule primordia of *Medicago sativa*, plasmodesmata among stele, pericycle, endodermis, and cortex cells were significantly more abundant compared to noninoculated root controls (Complainville et al., 2003). In *D. glomerata* root nodules induced by *Frankia*

spp., extensive plasmodesmal connections were observed between all cell types of the nodule, including mature infected cells (Schubert et al., 2011).

Suc is the primary form of photosynthetic carbon exported via the phloem from source tissues to various sinks, including root nodules (Vance et al., 1998; Lalonde et al., 2004; Ayre, 2011). Suc breakdown in root nodules, and more generally in higher plants, is mediated by Suc synthases and invertases (Morell and Copeland, 1984; Winter and Huber, 2000). Nodule-upregulated genes encoding Suc synthases and invertases have been isolated and characterized in several legumes and one actinorhizal species (Küster et al., 1993; Gordon and James, 1997; Gordon et al., 1999; Baier et al., 2007; Horst et al., 2007; Welham et al., 2009; Schubert et al., 2011). Suc synthase is necessary for efficient N-fixation in *Pisum sativum* (Gordon et al., 1999), *M. truncatula* (Baier et al., 2007), and *Lotus japonicus* (Horst et al., 2007).

Sugar uptake studies using nodule cell protoplasts isolated from broad bean revealed that uninfected protoplasts, but not those containing rhizobia, were able to import Suc and Glc, in a proton symport-dependent manner (Peiter and Schubert, 2003).

To our knowledge, the first nodule-enhanced sugar transporter to be described was *LjSUT4* in *L. japonicus* (Flemetakis et al., 2003). SUTs are a family of Suc/proton symporters within the Major Facilitator Superfamily and *LjSUT4* was found to be expressed in vascular bundles, inner cortex, and infected and uninfected cells of nodules. Later, it was localized to the

tonoplast and was shown to transport a range of sugars including Suc and maltose (Reinders et al., 2008). A proposed function for LjSUT4 is efflux of sugars stored in the vacuole for use in the cytoplasm. Another sugar transporter induced in N-fixing nodules, *DgSTP1*, was characterized in the nonlegume, *D. glomerata* (Schubert et al., 2011). *DgSTP1* belongs to the sugar porter (SP) family and has the highest relative uptake rate for Glc. It is also capable of transporting Gal, Xyl, and Man, albeit at much lower rates. Because of the specific increase of *DgSTP1* transcripts in infected nodule cells and an unusually low pH optimum of the protein, it has been suggested to fulfill the function of Glc export toward symbiotic bacteria prior to the onset of N-fixation (Schubert et al., 2011).

A porter family of sugar transporters, called SWEET, was discovered recently (Chen et al., 2010, 2012). Plant SWEET-genes are up-regulated in pathogenic interactions with bacteria and fungi where they are believed to transport sugars to the microbes (Yang et al., 2006; Ferrari et al., 2007; Antony et al., 2010). It has been suggested that members of this family may perform similar functions in mutualistic associations, given the fact that the nodule-specific gene, *MtN3* (designated *MtSWEET15c* in this study; Fig. 1), discovered almost 20 years ago (Gamas et al., 1996), was recently shown to be a member of the SWEET family (Chen et al., 2010;

Eom et al., 2015). The SWEET family is subdivided into four clades. Members of the SWEET family capable of Suc transport fall into Clade III, and have been localized primarily to the PM (Chen et al., 2012; Lin et al., 2014). Two Clade III SWEET transporters from rice were shown to be directly regulated by bacterial transcription activator-like effectors (Antony et al., 2010). They are thought to be involved in Suc efflux to the apoplast for consumption by pathogens, as mentioned above (Yang et al., 2006; Antony et al., 2010).

Here we describe, to our knowledge, the first functional characterization of a SWEET transporter in legume nodules, *MtSWEET11* from *M. truncatula*.

RESULTS

MtSWEET11 Is a Nodule-Specific Transporter in Clade III of the SWEET family

We conducted a systematic search for SWEET genes in the *M. truncatula* genome. Using IMGAG v4.0 of the genome, we identified 26 genes with high similarity to known SWEET transporter genes from Arabidopsis (Fig. 1). To identify symbioses-related SWEET genes, we analyzed the expression patterns of these genes in plants, using the *M. truncatula* Gene Expression Atlas (MtGEA v3; Benedito et al., 2008). One of the genes, *Medtr3g098930* (probeset Mtr.20845.1.S1_at; Affymetrix, Santa Clara, CA), was found to be expressed in a nodule-specific manner (Supplemental Fig. S1). Transcript levels of this gene in nodules 6 d post-inoculation (6 dpi) were 228-fold higher than in roots. Two different names of the gene have been suggested recently: *MtSWEET13* (Breakspear et al., 2014); and *MtSWEET11* (Lin et al., 2014). In this article we follow the naming system from the latter study. Secondary structure prediction for the *MtSWEET11* protein indicated seven putative transmembrane domains (Supplemental Fig. S2). Phylogenetic analysis of the *MtSWEET11* protein (Fig. 1) placed it into Clade III of the SWEET family with AtSWEET9 to AtSWEET15 of Arabidopsis (Chen et al., 2012), as well as OsSWEET11 and OsSWEET14 of rice (Yang et al., 2006; Antony et al., 2010), all of which are capable of Suc transport (Chen et al., 2012).

MtSWEET11 Is a Suc-Specific Transporter

To characterize the transport activity of *MtSWEET11*, we co-expressed the coding sequence of *MtSWEET11* with the high-sensitivity Förster resonance energy transfer (FRET) Suc sensor, FLIPsuc90 μ Δ 1V (Lager et al., 2006), in human embryonic kidney (HEK) 293T cells, which have low endogenous Suc uptake activity (Chen et al., 2012). *MtSWEET11* expression enabled HEK293T cells to accumulate Suc, as evidenced by a Suc-induced negative FRET ratio change (Fig. 2). Under the same test conditions, but using a Glc FRET sensor, Glc was not imported by *MtSWEET11* (Supplemental Fig. S3).

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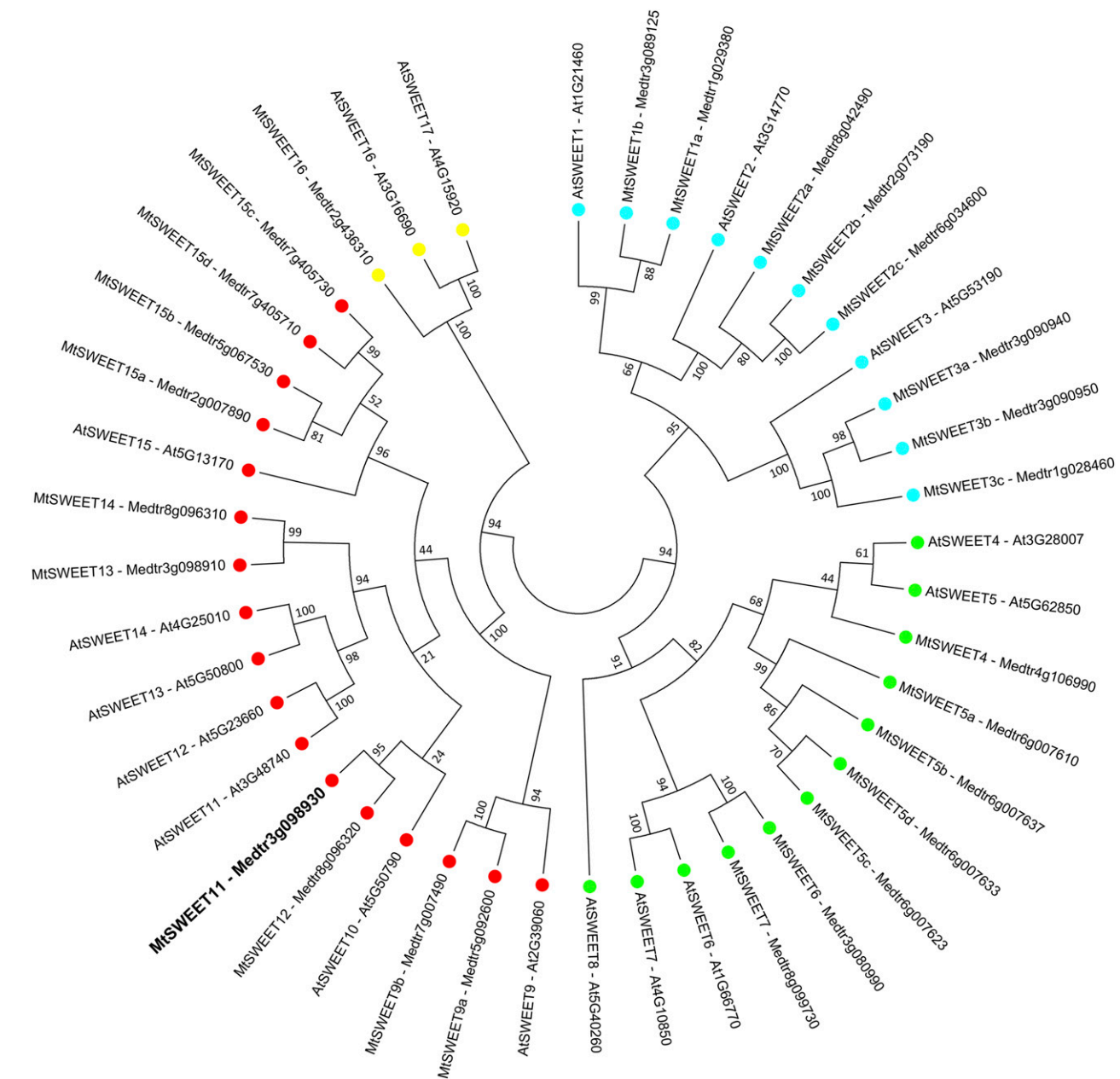


Figure 1. Phylogenetic tree of 26 *M. truncatula* and 17 Arabidopsis SWEET family members. The tree was constructed with MEGA 5.1 (Tamura et al., 2011), using protein sequences from *M. truncatula* (IMGAG v4) and Arabidopsis (<https://phytozome.jgi.doe.gov/pz/portal.html>). Tree branch values are bootstrap values (1000 reiterations). The tree demonstrates that MtSWEET11 falls into Clade III of SWEET transporters together with seven Arabidopsis homologs (AtSWEET9–15). Color code of Clades I–IV: blue, I; green, II; red, III; yellow, IV.

MtSWEET11 Is Expressed in Rhizobia-Infected Root Hair Cells and the Meristem, Invasion Zone, and the Distal Portion of the Vasculature of Nodules

MtSWEET11 is upregulated in nodules (Supplemental Fig. S1) and in root hairs of wild-type and *skl* plants inoculated with *S. meliloti* compared to plants inoculated with non-Nod factor producing control rhizobia (Breakspear et al., 2014). This suggests that MtSWEET11 expression is correlated with successful rhizobial infection.

We tested this hypothesis by using the same system to measure MtSWEET11 expression in root hairs of *S. meliloti*-inoculated *nin* plants. Expression of MtSWEET11 was significantly reduced in root hairs of *nin* seedlings relative to the wild-type controls (Fig. 3A). To further investigate MtSWEET11 gene expression patterns, we measured transcript levels of this gene in roots and in nodules of genotypes A17 and R108 at different stages of development and in different nodule zones, using

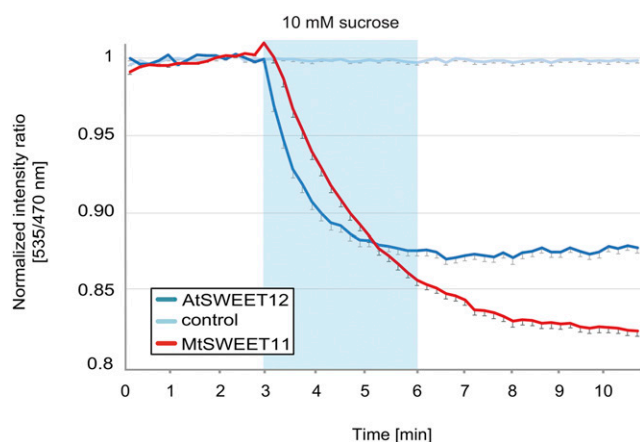


Figure 2. Identification of Suc as a substrate of MtSWEET11. Suc uptake was measured in HEK293T cells expressing *MtSWEET11* together with the cytosolic FRET Suc sensor FLIPsuc90 Δ 1V. Individual cells were analyzed by quantitative ratio imaging of CFP and Venus emission (acquisition interval 10 s). HEK293T/FLIPsuc90 Δ 1V cells were perfused with medium, followed by a pulse of 10 mM Suc. HEK293T cells transfected with sensor only (“control”, light blue) served as a negative control. Cells containing the sensor and Arabidopsis SWEET12 (“AtSWEET12”, dark blue) constituted a positive control. Similar to AtSWEET12, MtSWEET11 showed clear Suc influx (“MtSWEET11”, red), indicated by the decrease in normalized intensity ratio in response to Suc application. Values represent mean $\pm$ SE, $n \geq 4$ repeated with comparable results at least three times.

quantitative real-time PCR (qRT-PCR) assays. *MtSWEET11* transcripts were barely detectable in wild-type R108 roots before inoculation and 2 dpi with rhizobia (Fig. 3B). Transcript levels increased significantly by 4 dpi and reached a peak at 6 dpi, where the expression was over 3000-fold higher than in noninoculated roots. A steady decline in the transcript abundance was observed at 8 dpi, 10 dpi, 15 dpi, and 21 dpi, although levels in nodules at 21 dpi were still about 1000-fold higher than in non-inoculated roots (Fig. 3B). To better define the spatial pattern of *MtSWEET11* expression in nodules, qRT-PCR was performed on five manually dissected developmental zones of mature, 28-dpi nodules from ecotype A17 (Fig. 3C). *MtSWEET11* transcript levels were highest in the nodule meristem followed by the invasion zone, and were virtually undetectable in the other three nodule zones (interzone, N-fixation zone, and senescence zone). To further visualize *MtSWEET11* expression in developing nodules, wild-type R108 roots were transformed with the GUS-reporter gene fused to 1705 bp of the *MtSWEET11* promoter and subsequently inoculated with *S. meliloti* strain Sm1021 containing a plasmid with the *lacZ*-reporter gene. *pMtSWEET11:GUS* expression was detectable as early as 1 dpi in dividing root cortical cells (Fig. 3D) underlying sites of rhizobia infection, indicated by Magenta-Gal staining for rhizobial LacZ activity. Further, strong GUS staining was found in actively dividing nodule primordia at 3 dpi (Fig. 3E). At 10 dpi and 21 dpi, *MtSWEET11* expression was associated predominantly with the meristem, the

invasion zone, and distal part of the vascular bundles (Fig. 3, F–I). Weak GUS signal was detected in the proximal and basal portions of the vascular system of 10-dpi nodules after prolonged (6 h) staining (Fig. 3G). No GUS signal was found in the interior of the N-fixation zone (Fig. 3, F–I), even when nodules were transversely sectioned prior to staining (results not shown).

MtSWEET11 Protein Is Associated with the Plasma Membrane of Uninfected Nodule Cells and with Infection Thread and Symbiosome Membranes of Infected Cells

In order to determine the possible subcellular location(s) of MtSWEET11 protein, we analyzed the distribution pattern of an MtSWEET11-GFP fusion protein expressed in plant cells. The fusion protein was transiently expressed under the control of the constitutive 35S promoter in tobacco epidermal cells, or the maize polyubiquitin promoter (Christensen et al., 1992) or the native promoter (1.7 kb) in *Medicago* nodules. Transformation of tobacco (*Nicotiana benthamiana*) leaf epidermal cells with *p35S:MtSWEET11-GFP* resulted in a fluorescent signal at the PM, but not at any other organelle membranes (Supplemental Fig. S5, A and B). Expression of *pUBI:MtSWEET11-GFP* in *Medicago* nodules resulted in green fluorescence predominantly at the PM of uninfected cortical cells and at internal structures of infected cells, but not in uninfected cells of the same tissue (Supplemental Fig. S5, C and D). Since this contrasted with our gene expression analysis, we tested whether the internal cellular location of MtSWEET11-GFP was an artifact of ectopic over-expression from the polyubiquitin promoter. For this we used the native *MtSWEET11* promoter to drive MtSWEET11-GFP expression in nodules. Green fluorescence associated with expression of *pMtSWEET11:MtSWEET11-GFP* in nodules was detected on transcellular infection threads (ITs; Fig. 4F) and symbiosomes in the infected cells, with no signal at the PM of either the nodule meristematic cells or cortical cells (Fig. 4, B, D, and F–H). Surprisingly, the protein localization pattern of MtSWEET11-GFP driven by the native *MtSWEET11* promoter did not match that of the GUS protein driven by the same promoter (Fig. 3, F–I). Therefore, we generated a control construct, in which the *MtSWEET11* promoter was fused to GFP alone. Green fluorescence resulting from expression of *pMtSWEET11:GFP* was observed only in the invasion zone and vascular bundles of transgenic nodules (Fig. 4, A, C, and E), consistent with the *pMtSWEET11:GUS* results.

Symbiotic Phenotype of *sweet11* Mutants

Two independent *sweet11* mutants were isolated from a *Tnt1*-insertional mutant population of *M. truncatula*, using a PCR-based approach (Tadege et al., 2008; Cheng et al., 2011). In lines NF12718 and NF17758, the *Tnt1* transposon is located in exon 3 at positions 160 bp

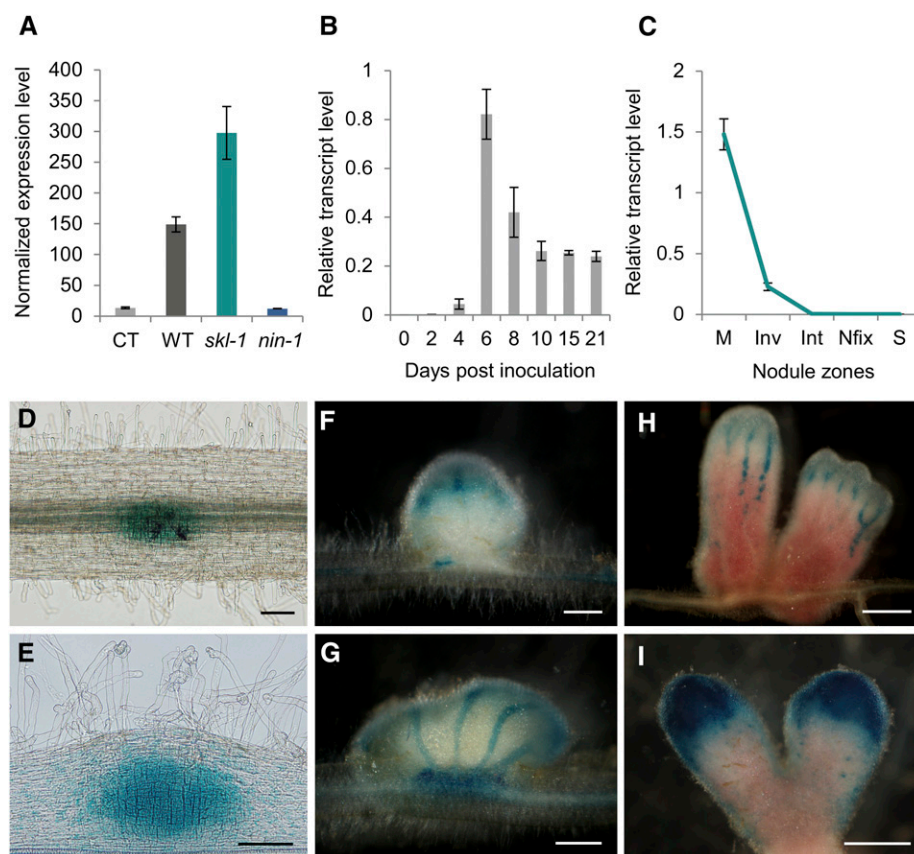


Figure 3. Expression of *MtSWEET11* is limited to root hairs of infected plants, the nodule meristem, the invasion zone, and the nodule vasculature. A, *MtSWEET11* expression in root hairs 5 dpi with *S. meliloti* 1021 in wild type (Jemalong A17) in comparison with *skl-1* and *nin-1* mutants. The control is wild-type 5 dpi with the non-Nod factor producing *S. meliloti* nodΔD1ABC (SL44). Data for wild type and *skl-1* from Breakspear et al. (2014). B, Time course of *MtSWEET11* expression in nodules (qRT-PCR). C, *MtSWEET11* expression in manually dissected nodule zones of a mature nodule (28 dpi) measured using the *Medicago* Genome Array (Affymetrix). Error bars in (A) represent mean $\pm$ SE values of three biological replicates. Error bars in B and C represent SD values of three biological replicates. CT: control. Nodule zones: M: meristem; Inv: invasion zone; Int: interzone; Nfix: N-fixation zone; S: senescence zone. D, E, F, G, H, I, Staining for GUS activity. (D) Nodule primordium ITs (1 dpi) showing rhizobia stained with Magenta-Gal (*lacZ*-reporter). (E) Nodule primordium (3 dpi). (F) Young nodule (10 dpi) after 1 h staining for GUS activity. (G) Young nodule (10 dpi) after 6 h staining for GUS activity. (H) Mature nodule (21 dpi) after 1 h staining for GUS activity. (I) Mature nodule (21 dpi) after 6 h staining for GUS activity. Bars: (D and E), 100 μ m; (F and G), 300 μ m; (H and I), 1000 μ m.

(*sweet11-1* allele) and 232 bp (*sweet11-2* allele), respectively, relative to the translational start of *MtSWEET11* (Supplemental Fig. S6).

Homozygous *sweet11-1* and *sweet11-2* mutants were obtained and phenotyped with respect to nodule primordia/ nodule number, leaf color, plant size, fresh weight, and nitrogenase activity (acetylene reduction assay). No significant differences between mutants and wild-type controls were found for these parameters, except for biomass, which was slightly lower in one mutant, but not the other (Supplemental Figs. S7–S9).

Given the biochemical function of *MtSWEET11* in Suc transport, we looked for conditional phenotypes of the *sweet11* mutants associated with altered Suc transport in nodules, under conditions where Suc supply to nodules was limited. The *sweet11* mutants, their wild-type-like siblings, and genotype R108 controls

were grown under low-light conditions, with 7% or 20% of normal light intensity. No significant difference in leaf color, plant size, fresh weight, or nitrogenase activity was found between the mutants and control plants (see Supplemental Fig. S9; it exemplifies the results for line NF12718). In all cases, low light reduced plant growth and N-fixation, as expected.

Loss of *MtSWEET11* Activity Is Not Associated with Transcriptional Activation of other Potential Suc Transporter Genes

In order to rule out the unlikely possibility that *Tnt1* was excised from *MtSWEET11* transcripts in mutant nodules to produce transcripts that encode functional transporter, we performed RT-PCR using *MtSWEET11*

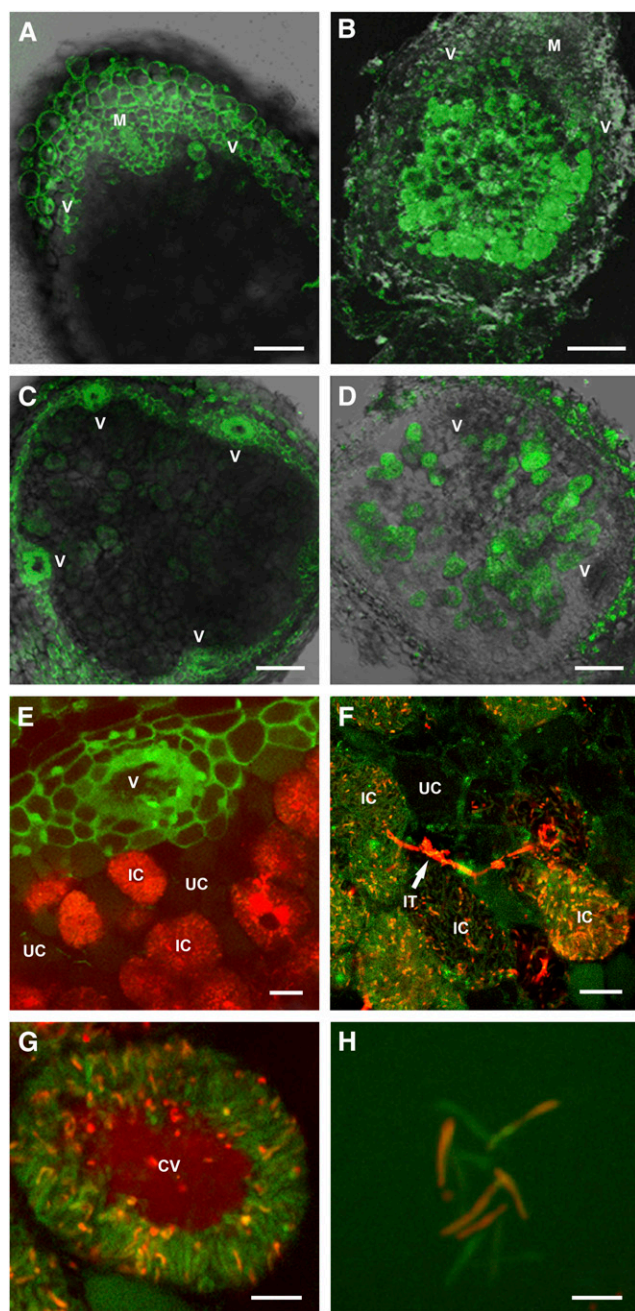


Figure 4. MtSWEET11-GFP fusion driven by the native promoter localizes to symbiosomes, ITs, and PM of non-infected nodule cells. Images were taken with a confocal laser scanning microscope, except for (H), which was acquired with a confocal spinning-disk microscope. A, C, and E, GFP driven by the *SWEET11* native promoter (control). B, D, F, G, and H, GFP fused to the C terminus of MtSWEET11 driven by the native promoter. (A–D) Overlay with bright-field images of transverse sections (A and B) and cross sections (C and D) of 12 dpi nodules. (E–H) Overlay with red fluorescence channel showing rhizobia (mCherry plasmid). M: nodule meristem; V: nodule vasculature; IC: infected cell; UC: uninfected cell; CV: central vacuole. G, Fully developed infected cell from N-fixation zone. Individual rod-shaped symbiosomes are visible. H, Symbiosomes released from a dissected cell. Bars: (A–D) 100 μ m; (E and F), 20 μ m; (G and H) 10 μ m. Note that localization pattern of GFP expressed from the native *MtSWEET11* promoter is virtually the same as seen using the corresponding GUS reporter in Figure 3.

coding sequence-specific primers (Supplemental Table S1) and nodule cDNA from wild-type R108 and the two mutant lines. As expected, no wild-type *MtSWEET11* transcript was present in the two mutants (Supplemental Fig. S10).

The absence of symbiotic defects in the *sweet11* mutants pointed to the existence of other nodule Suc transporters that can compensate for the loss of MtSWEET11 activity in these mutants. Several *SWEET* genes of clade III and other clades were found in the *M. truncatula* Gene Expression Atlas (MtGEA v3) data to be expressed in nodules, including *MtSWEET1b* (*Medtr3g089125.1*), *MtSWEET2b* (*Medtr2g073190.1*), *MtSWEET3c* (*Medtr1g028460.1*), *MtSWEET12* (*Medtr8g096320.1*), and *MtSWEET15c* (*Medtr7g405730.1*; Supplemental Dataset 3; Benedito et al., 2008). Likewise, members of the unrelated *SUT* family of Suc transporter genes and several genes from the *SP* family were expressed in nodules, including *MtSUT1-1* (*Medtr1g096910*), *MtSUT4-1* (*Medtr5g067470*; Reinders et al., 2008), *MtSUT4-2* (*Medtr3g110880*), and *MtSTP13* (*Medtr1g104780*; Supplemental Dataset 3). Some of these genes were induced during nodule development, including *MtSWEET1b* (*Medtr3g089125.1*), *MtSWEET15c* (*Medtr7g405730.1*), *MtSUT4-2* (*Medtr3g110880*), and *MtSTP13* (*Medtr1g104780*; Supplemental Dataset 3). We confirmed these result for genes *MtSWEET1b* (*Medtr3g089125.1*), *MtSWEET12* (*Medtr8g096320.1*), *MtSWEET15c* (*Medtr7g405730.1*), *MtSUT4-1* (*Medtr5g067470*), *MtSUT4-2* (*Medtr3g110880*), and *MtSTP13* (*Medtr1g104780*) by qRT-PCR (Supplemental Fig. S11). However, none of these genes was induced in *sweet11* mutant nodules in response to loss of MtSWEET11. Analysis of the laser-capture microdissection data for different nodule zones from Roux et al. (2014) indicates that *Medtr3g090950* and *Medtr6g007623* are also expressed in nodules. In summary, transcripts of several putative Suc transporter genes are present in nodules that could potentially fulfill the role(s) of MtSWEET11 in its absence, although none of the genes tested were expressed at higher levels in the *sweet11* mutants than in the wild type.

DISCUSSION

Previous studies have demonstrated the importance of Suc metabolism in nodules for symbiotic N-fixation (e.g. Baier et al., 2007; see Vance 2008 for a review). Suc is delivered to nodules via the phloem (Vance et al., 1998) and distributed to other cell types via apoplasmic and/or symplasmic routes. Uptake of Suc from the apoplasm requires specific transport proteins in the PM (see Ayre 2011, for a review). Movement of Suc between compartments of individual cells also requires transporters in the intervening membranes. Many putative Suc transporter genes have been identified in legumes and are expressed in nodules (Wienkoop and Saalbach, 2003; Benedito et al., 2010; Limpens et al., 2013; Clarke et al., 2015), although none of these have been characterized genetically. In this study, we

characterized a nodule-specific Suc transporter of *M. truncatula*, MtSWEET11 at the molecular, biochemical, cellular, and genetic levels.

MtSWEET11 belongs to Clade III of the SWEET protein family (Fig. 1), a branch of the family that is thought to specialize in Suc transport (Chen et al., 2012; Lin et al., 2014; see Eom et al., 2015, for a review). Our experiments confirmed the substrate specificity of MtSWEET11, which transported Suc but not Glc when expressed in HEK293T cells (Fig. 2, Supplemental Fig. S3).

MtSWEET11 promoter analysis using either *GUS* or *GFP* reporter genes expressed in transformed roots and nodules indicated that MtSWEET11 expression is limited to the nodule apex (meristem and infection zone) and to the vascular system (Figs. 3, F–I, and 4, A, C, and E). This spatial pattern of gene expression was supported by quantitative measurements of MtSWEET11 transcript levels, which were found to be highest in the meristem followed by the invasion zone, with relatively little expression in other zones of mature, N-fixing nodules (Fig. 3C). Our transcript data are consistent with the expression pattern revealed in the RNAseq-based study of Roux et al. (2014), where MtSWEET11 was expressed most strongly in the nodule apex, including the distal invasion zone and the meristem (Supplemental Dataset 3 and Supplemental Dataset 4). Maximal expression of MtSWEET11 was found in young nodules, 6 dpi, which declined in older nodules but remained approximately 1000-fold higher in nodules than in roots (Fig. 3B). The temporal and spatial patterns of MtSWEET11 gene expression indicate that the encoded transporter plays roles in Suc transport during the first few days of the symbiosis with rhizobia, in infected root hair cells, and underlying cortical cells, as well as throughout nodule development and during symbiotic N-fixation in mature nodules.

Localization of the MtSWEET11 protein using an MtSWEET11-GFP fusion protein expressed in genetically transformed cells indicated a PM location in uninfected cells and different locations in infected cells, namely IT and symbiosome membranes (SM) (Fig. 4). Differential localization of membrane proteins in infected versus uninfected cells is not unprecedented in plant-microbe interactions and has been observed for the P-transporter, MtPT4 during arbuscular mycorrhizal symbiosis in *Medicago* (Pumplin et al., 2012), and potato proteins that are rerouted to the plant-pathogen interface in the heterologous expression system, *N. benthamiana*-*Phytophthora infestans* interaction (Bozkurt et al., 2015). Taken together with the gene expression data described above, the protein localization data indicate that MtSWEET11 plays different roles in Suc transport in different cell types. In uninfected cells, where MtSWEET11 is located in the PM, the transporter probably plays a role in Suc uptake from the apoplast. Based on our MtSWEET11 promoter-GUS/GFP results, apoplastic Suc uptake via MtSWEET11 appears to be important in vascular tissues, which are the primary conduit for Suc entry into nodules. Suc taken up by cells

within or surrounding the vascular tissues may subsequently move to adjacent and distant cells via symplasmic Suc transport through plasmodesmata, which are prevalent in nodule cells (Complainville et al., 2003). Nonetheless, MtSWEET11 expression in cells of the meristem indicates that these cells are probably also able to take up Suc from the apoplast, presumably to fuel hyperactive metabolism and cell division.

MtSWEET11 expression is induced in root hairs shortly after contact with rhizobia and its expression is dependent on the transcription factor NIN (Fig. 3A; Supplemental Fig. S4; Breakspear et al., 2014). This regulation by NIN could be direct, but may also be indirect as *nin* mutants cannot form ITs (Marsh et al., 2007). Given the apparent location of MtSWEET11 in transcellular ITs of cortical cells, it seems reasonable to speculate that MtSWEET11 resides in the IT membrane of infected root hair cells as well, although we were unable to observe it there perhaps because of relatively low levels of expression of the gene in those cells. In any case, the IT membrane is contiguous with the PM, so lateral movement of MtSWEET11 from the PM to the IT membrane is conceivable. An IT membrane localization of MtSWEET11 would put it in a strategic location to supply Suc to rhizobia within ITs. In this context, it is salient to note that SWEET transporters in other plant species are induced by pathogens to ensure their sugar supply (Yang et al., 2006; Antony et al., 2010). Since purified Nod-factors from rhizobia are insufficient to induce full-scale MtSWEET11 expression in root hair cells (Supplemental Fig. S4; Breakspear et al., 2014), it would be interesting to determine whether other bacterial factors are involved. Although dicarboxylic acids are believed to be the primary source of carbon for mature, N-fixing bacteroids in nodules (Udvardi and Poole, 2013; Benedito et al., 2010), it is possible that other C-compounds that are common in root exudates, such as sugars and amino acids (Hirsch et al., 2013), could be provided to rhizobia in ITs (analogous to the apoplast) prior to their release into cells within symbiosomes. Expression data from Roux et al. (2014) based on laser capture microdissection of cells from different nodule zones show expression of *agl*, *thu*, and *frs* rhizobial genes involved in the uptake and/or catabolism of Suc (Willis and Walker, 1999), trehalose (Jensen et al., 2002), and Fru (Lambert et al., 2001) in the nodule apex, suggesting the importance of Suc and derived sugars as energy sources for rhizobia contained within the IT. It would be interesting to determine whether sugar supply to rhizobia is reduced in *sweet11* mutants, perhaps using a sugar-sensitive reporter gene in rhizobia.

It came as a surprise to us that MtSWEET11-GFP fusion protein expressed from the MtSWEET11 promoter accumulated in cells of the N-fixing zone (Fig. 4), given that free GFP or GUS expressed from the same promoter accumulated in the meristem and invasion zones and in vascular bundles (Figs. 3, F–I, and 4, A, C, and E), locations consistent with the transcript data (Fig. 3C, Supplemental Dataset 3, Supplemental Dataset 4).

Given the congruence among the three other types of data, we conclude that MtSWEET11-GFP localization in nodules does not accurately reflect *MtSWEET11* gene expression, and we speculate that intercellular movement of the SWEET-GFP protein may account for its unexpected distribution in nodules. There are precedents for membrane proteins accumulating in cells other than those that express the corresponding genes (Kühn et al., 1997).

Our MtSWEET11-GFP fusion protein experiments point to possible locations for MtSWEET11 on the PM of uninfected cells and the SM of infected cells in the N-fixing zone of nodules (Fig. 4, F–H). Sugar uptake data from infected or uninfected cells isolated from broad bean nodules, indicated that uninfected cells were competent in Suc uptake while infected cells were not (Peiter and Schubert, 2003). It would be interesting to obtain similar data for cells isolated from *sweet11* mutant nodules, to determine whether MtSWEET11 contributes to Suc uptake in uninfected nodule cells.

What are the implications of the possible SM location of the MtSWEET11 protein? As for an IT location, a SM location would place MtSWEET11 in a position to transport Suc toward the rhizobia within symbiosomes. Although sugars are unlikely to be primary sources of carbon for N-fixing bacteroids (Udvardi and Poole, 2013), Suc and Glc have been found in the symbiosome space of soybean nodules, at concentrations of 75.4 nmol g nodule FW⁻¹ and 37.7 nmol g nodule FW⁻¹, respectively, which corresponds to 6% and 3% of the total sugar in the symbiosome space, respectively. (Tejima et al., 2003). Consistent with this, the rhizobial *aglEFGAK* operon encoding the Suc-inducible α -glucoside transport system, although mainly expressed in the nodule apex, is significantly expressed in the N-fixation zone (Roux et al., 2014; Mauchline et al., 2006; Jensen et al., 2002). It would be interesting to determine whether Suc levels in the symbiosome space of *sweet11* mutants are lower than those of the wild type. Proteomic analysis of SM purified from nodules of *L. japonicus* revealed the presence of a putative Suc transporter of the SUC family (Wienkoop and Saalbach, 2003). Thus, if MtSWEET11 is active on the SM of *Medicago* nodules, it may not be the only active Suc transporter there. This leads us to the subject of functional redundancy of Suc transporters in *Medicago* nodules.

Loss of MtSWEET11 function in *sweet11* mutants had no significant effect on nodulation or N-fixation under conditions tested in this study (Supplemental Figs. S7–S9). Although it is possible that subtle conditional phenotypes may be found for the *sweet11* mutants in further studies, our results indicate that other Suc transporters are active in *Medicago* nodules and can perform the role(s) of MtSWEET11 in its absence. Several SWEET genes of clade III and other clades are expressed in nodules, including *MtSWEET1b* (*Medtr3g089125.1*), *MtSWEET2b* (*Medtr2g073190.1*), *MtSWEET3c* (*Medtr1g028460.1*), *MtSWEET12* (*Medtr8g096320.1*), and *MtSWEET15c* (*Medtr7g405730.1*; Supplemental Dataset 3; Benedito et al., 2008). In fact, analysis of the entire SWEET

family across nodule zones indicates that although *MtSWEET11* accounts for 75% of SWEET gene expression in the nodule apex, it only accounts for 8% of the total reads in the N-fixation zone (data from Roux et al., 2014; Supplemental Dataset 4). Likewise, members of the unrelated SUT family of Suc transporter genes and several genes from the SP family are expressed in nodules, including *MtSUT1-1* (*Medtr1g096910*), *MtSUT4-1* (*Medtr5g067470*; Reinders et al., 2008), *MtSUT4-2* (*Medtr3g110880*), and *MtSTP13* (*Medtr1g104780*; Supplemental Dataset 3). Clearly, there are many putative Suc transporters expressed in nodules, presumably a reflection of the importance of Suc and Suc transport for legume nodule metabolism.

MATERIALS AND METHODS

Identification of SWEET Family Members in *Medicago truncatula*

SWEET family genes were identified by sequence similarity to 17 *Arabidopsis* SWEETs, using the BLASTN algorithm at the IMGAG v4 coding sequence (CDS) database (<http://bioinfo3.noble.org/doblast/>). The phylogenetic tree was constructed with MEGA 5 software (Tamura et al., 2011). *MtSWEET11* (IMGAG v4 *Medtr3g098930.1*; *Medicago Truncatula* Genome Project v4.0, J. Craig Venter Institute, Rockville, MD) was identified as nodule-specific based on data in the *M. truncatula* Gene Expression Atlas (Benedito et al., 2008).

Plant Growth Conditions

Seeds of *M. truncatula* ecotype R108 and mutant plants were scarified with concentrated H₂SO₄ for 8 min, rinsed five times with sterile water, and sterilized for 3 min with a 33% (v/v) solution of commercial bleach (6% w/v of Cl) containing 0.1% (v/v) TWEEN 20. Seeds were then rinsed 10 times with sterile water and left in the dark in 50 mL Falcon tubes overnight, at room temperature, to imbibe. After transfer to sterile water-soaked filter paper in a petri dish, seeds were incubated in the dark for 2 d at 4°C and then for 2 d at room temperature. Seedlings were transferred to a 1:2 mixture of vermiculite/turface (washed and double-autoclaved) in plastic cones and placed in a walk-in growth chamber set at 200 $\mu\text{E m}^{-2} \text{s}^{-1}$ for 16 h light, dark period 8 h, 21°C day/night, and 40% humidity. Up to the day of inoculation with rhizobia, plants were supplied only with distilled water. Seven days after germination, plants were inoculated with a 20–24 h culture of *Sinorhizobium meliloti* Sm1021 (*OD*₆₀₀ 0.75–1.20, TY medium) resuspended in 2x zero-nitrogen half-strength B&D medium (Broughton and Dilworth, 1971) to *OD*₆₀₀ = 0.02. Each plant was supplied with 50 mL of the inoculum to the surface of the soil by the stem. Any remaining suspension was added to the tray. Subsequently, distilled water was added to the tray every 4 d up to the point of harvesting. Thus, atmospheric nitrogen (N)-fixation was the only controlled source of nitrogen for these plants. They were not exposed to N-containing fertilizers or N-rich soil at any stage before the measurements. For seed production, plants inoculated with rhizobia were transferred from vermiculite/turface to soil 14 dpi and cultivated in a greenhouse. In the light-limitation experiment, on the day of inoculation plants were covered with domes made of commercial antimosquito net (Charcoal Fiberglass Screen Cloth; NY Wire, Hanover, PA) to reduce available light down to 7% and 20% of normal (three and two layers of the net, respectively). Control plants (100% light) were left uncovered.

Isolation of *Tnt1* Mutant Lines for *MtSWEET11*

Reverse genetic screening of a *Tnt1* mutant population of *M. truncatula* (Tadege et al., 2008) for insertions in the *MtSWEET11* gene was carried out as described previously in Cheng et al., (2011). Oligonucleotide sequences used as primers for PCR and genotyping of *sweet11-1* and *sweet11-2* insertion alleles are provided in Supplemental Table S1. To reduce the number of unlinked *Tnt1* insertions in *sweet11* mutants, the mutants were back-crossed twice to the wild-type R108 lines as described in Taylor et al. (2011).

Construction of DsRed-Encoding Destination Vectors pUBICGFP-DR and pRRcGFP for Protein Localization with a C-Terminal GFP Tag

To enable red fluorescence-based selection of transformed roots, we modified commercial vectors pMBb7Fm21GW-UBIL (VIB) and pKGW RedRoot (VIB). A 2515-bp region containing the DsRed cassette of pKGW RedRoot was PCR-amplified with primers DsRED-F-*KpnI* and DsRED-R-AvrII (Supplemental Table S1), cut with *KpnI* and *AvrII* and ligated to adapters converting *KpnI* and *AvrII* sites into *AflIII* and *SacI* sites, respectively. The adapters were formed from oligonucleotides supplied with 5' (*AflIII*-*KpnI*-S/AS and *SacI*-*AvrII*-S/AS; Supplemental Table S1). This DsRed product was introduced into *AflIII*/*SacI*-cut pMBb7Fm21GW-UBIL in place of 1663 basepairs containing p35S and BAR, which were eliminated to make the resulting vector, pUBICGFP-DR, shorter. A 950-bp region containing the GFP gene and terminator was amplified from pK7FWG2 (VIB) with primers GFP-F-*SpeI*/T35S-R-*HindIII* (Supplemental Table S1), digested with *SpeI* and *HindIII* and ligated into pKGW RedRoot pre-cut with the same restriction endonucleases, to produce vector pRRcGFP.

Cloning the CDS and Promoter Region of *MtSWEET11*

Primers for PCR-based cloning of *MtSWEET11* CDS from *M. truncatula* ecotype R108 (Supplemental Table S1) were designed on the basis of a partial genome sequence assembly performed at the Samuel Roberts Noble Foundation (Ardmore, OK). The predicted R108 *MtSWEET11* CDS differed by 6 bp from that of ecotype Jemalong A17 deposited at the International *Medicago* Genome Annotation Group database (IMGAG v4). This was later confirmed by sequencing of the CDS amplified from R108 cDNA.

cDNA produced from mRNA of 21-dpi root nodules of *M. truncatula* ecotype R108 served as template for PCR-amplification of the 810-bp *MtSWEET11* CDS with primers SW11-CDS-F and SW11-CDS-R (Supplemental Table S1), the latter of which was designed to eliminate the stop codon. The PCR product was cloned into pDONR207 (Life Technologies/Thermo Fisher Scientific, Guilford, CT). For substrate transport assays in HEK293T cells, the entry clone was recombined with pcDNA3.2/V5-DEST (Life Technologies/Thermo Fisher Scientific). For constitutive expression of *MtSWEET11*, the CDS was cloned into destination vector pUBICGFP. A quantity of 1705 bp of the promoter region upstream of the translational start codon was amplified from *M. truncatula* ecotype Jemalong A17 (primers SW11-pro-F and SW11-pro-R, Supplemental Table S1), cloned into pDONR207, and recombined with destination vector pKGWFS7.0 (VIB) for GUS assays. For subcellular localization of the *MtSWEET11* protein, the 1705-bp promoter fragment was fused to the *MtSWEET11* CDS by overlap extension-PCR (Higuchi et al., 1988). Individual elements for the fusion were amplified from corresponding templates (first-stage PCR) with primers SW11-pro-F/SW11-fus-R from genomic DNA and SW11-fus-F/SW11-CDS-R from the expression clone. The products were mixed in 1:1 m and used as an overlapping template for the second-stage PCR that involved primers SW11-pro-F and SW11-CDS-R alone (see Supplemental Table S1 for primer sequences). The resulting fusion was cloned into pDONR207 and recombined with a destination vector pRRcGFP. In both destination vectors, the GFP CDS was located 3' to the *MtSWEET11* CDS. All clones were verified by sequencing in both directions.

Transport Assays with HEK293T Cells

Suc and Glc uptake assays in HEK293T cells were conducted as described in Chen et al. (2012).

Root Transformation, Histochemical Staining, and Light Microscopy

Plants with composite, transgenic roots were generated using an axenic *Agrobacterium rhizogenes* ARqua1-mediated procedure described in the *Medicago truncatula* Handbook (<http://www.noble.org/Global/medicagohandbook>) without antibiotic selection. To enable visualization of rhizobia in roots and nodules, *S. meliloti* strain Sm1021 was transformed with the *lacZ*-reporter plasmid *pXLGD4* (Boivin et al., 1990). Inoculation with rhizobia was conducted two weeks after transfer of plants to substrate. For localization of promoter-GUS activity, entire root systems were harvested at different time points and stained with X-Gluc (Gold Biotechnology, Olivette, MO), as described in Boivin

et al. (1990). Roots were also stained with Magenta-Gal (Gold Biotechnology) for *lacZ*-activity, as described in Arrighi et al. (2008). Whole-nodule images were taken with a model no. SMZ 1500 fluorescent stereo microscope using a model no. RS Ri1 digital camera (both by Nikon, Melville, NY). *pMtSWEET11::GUS* fusion experiments were conducted in three biological replicates, with approximately 24 plants transformed in each replicate. Sections of GUS-stained nodules (50 μ m) were obtained with a Vibratome 1000 Plus (Technical Products International, St. Louis, MO). A model no. BX14 light microscope, equipped with a model no. DP72 digital camera (both by Olympus, Melville, NY), was used to visualize the sections. Localization of proteins expressed under the control of the ubiquitin promoter was performed in two biological replicates. Native promoter *MtSWEET11*-GFP and GFP-only localization experiments were performed in four and two biological replicates, respectively. Each time, approximately 24 plants were transformed. Five or more nodules with the strongest DsRed signal (transformation marker) were collected under the RFP channel 10–12 dpi and handmade sections were prepared. Images were acquired with a TCS SP2 AOBS confocal laser scanning microscope with LCS Lite software (both by Leica Microsystems, Wetzlar, Germany) or an UltraVIEW spinning disc confocal microscope supplied with Velocity 6.1.1 software package (both by PerkinElmer, Waltham, MA). Digital images were processed with Adobe Photoshop CS4 (Adobe Systems, San Jose, CA).

Phenotypic Analysis and Acetylene Reduction Assay

Visual assessment of nodules, shoots, and roots of wild-type and mutant plants was made at 10, 21, and 42–44 dpi. Images of representative plants were acquired with a model no. DS126131 EOS 30D digital camera (Canon, Tokyo, Japan). Fresh weight of whole plants was recorded with analytical scales. Entire root systems of 42-dpi plants were dissected and placed into sealed test tubes for acetylene reduction assays. Gas measurements were performed with a model no. 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) after 18–24 h of incubation with 10% (v/v) acetylene at 28°C, as described previously in Oke and Long (1999).

Nodule and Root Hair Collection, RNA Extraction, and cDNA Synthesis

For the nodulation time course (0–21 dpi), we used cDNA generated by Sinharoy et al., (2013). A quantity of 0–4 dpi samples were collected from the root zone susceptible to nodulation, whereas individual nodules were collected for samples of 6 dpi and onwards. For gene expression profiling in different nodule zones, 28-dpi nodules (Jemalong A17) were dissected into the meristematic zone, invasion zone, interzone II-III, N-fixation, and senescence zone. Leghemoglobin coloration and overall nodule morphology were used to discriminate between different nodule zones. Hand-dissection was performed on unfixed nodules, under a dissecting microscope. Three independent biological replicates, each consisting of multiple samples, were prepared for each nodule zone. qRT-PCR and hybridization to the GeneChip *Medicago* Genome Array (Affymetrix, Santa Clara, CA) were performed on each of these biological replicates. Root hairs were removed from 10-d-old *nin-1* seedlings 5 dpi with *S. meliloti* 1021 and RNA was isolated for microarray analysis as previously described in Breakspear et al. (2014). Data were normalized with data from Breakspear et al. (2014): *skl-1* 5 dpi with *S. meliloti* 1021; wild-type (Jemalong A17) plants 5 dpi with *S. meliloti* 1021, and wild-type 5 dpi with *S. meliloti* nodΔD1ABC (SL44). Nodules from wild-type and mutant plants were collected 8 dpi (*S. meliloti* Sm1021), immediately frozen in liquid nitrogen, and stored at –80°C. Total RNA of frozen nodules and nodule zones was extracted with TRIZOL reagent (Life Technologies), as described in Chomczynski and Mackey (1995). Isolated RNA was digested with RNase-free DNase I (Ambion, Naugatuck, CT), following manufacturer's recommendations, and further column-purified with an RNeasy MinElute CleanUp kit (Qiagen, Hilden, Germany). RNA was quantified using a model no. ND-100 spectrometer (NanoDrop Technologies, Wilmington, DE) and the purity assessed with a Bioanalyzer 2100 (Agilent Technologies). Nodule-derived cDNA was synthesized with SuperScript III Reverse Transcriptase (Life Technologies), using oligo(dT)20 or Random Hexamers (Thermo Fisher Scientific) to prime the reaction as described previously in Kakar et al. (2008). cDNA synthesis was performed individually for three biological replicates each corresponding to 17–20 plants. Hybridization to the *Medicago* Genome Array was conducted following the manufacturer's instructions (Affymetrix). Microarray data were analyzed as previously described in Benedito et al. (2008).

qRT-PCR Analysis

PCR reactions were carried out in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). Reaction mixtures were set up in an optical 384-well plate so that a 5- μ L reaction contained 2.5 μ L SYBR Green Power Master Mix reagent (Applied Biosystems), 15 ng cDNA, and 200 nM of each gene-specific primer. Amplification of templates followed the standard PCR protocol: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min; and SYBR Green fluorescence was measured continuously. Melting curves were generated after 40 cycles by heating the sample up to 95°C for 15 s followed by cooling down to 60°C for 15 s and heating the samples to 95°C for 15 s. Transcript levels were normalized using the geometric mean of three housekeeping genes, *MtPI4K* (Medtr3g091400), *MtPTB2* (Medtr3g090960), and *MtUBC28* (Medtr7g116940). qRT-PCR was performed for three biological replicates and displayed as relative expression values. To ensure specificity of primers to each *MtSWEET* gene subjected to qRT-PCR, all primer pairs (14 pairs) were tested on four cDNA libraries (Supplemental Fig. S11), which yielded melting curves corresponding to unique products in each case.

Statistical Analysis

Statistical significance of observed differences was determined with a Student's *t*-test implemented using the T.TEST function in Excel (Microsoft, Redmond, WA) with two-tailed distribution and unequal variances.

Accession Numbers

Supplemental Dataset 1 contains IMGAG v4.0 gene identifiers and coding sequences of genes used in this study, as well as corresponding National Center for Biotechnology (NCBI; <http://www.ncbi.nlm.nih.gov/>) accession numbers. Absence of the accession number indicates sequences not present in the database. Accession numbers of genes with IMGAG v4.0 sequences different from NCBI entries are shown in brackets. For members of *MtSWEET* family, Transporter Classification Database family numbers are also shown. The CDS of *MtSWEET11* from *M. truncatula* ecotype R108 is available from NCBI under accession no. KF998083. Genes and corresponding IMGAG v4.0 gene IDs are as follows: *MtSWEET1a* (Medtr1g029380), *MtSWEET1b* (Medtr3g089125), *MtSWEET2a* (Medtr8g042490), *MtSWEET2b* (Medtr2g073190), *MtSWEET2c* (Medtr6g034600), *MtSWEET3a* (Medtr3g090940), *MtSWEET3b* (Medtr3g090950), *MtSWEET3c* (Medtr1g028460), *MtSWEET4* (Medtr4g106990), *MtSWEET5a* (Medtr6g007610), *MtSWEET5b* (Medtr6g007637), *MtSWEET5c* (Medtr6g007623), *MtSWEET5d* (Medtr6g007633), *MtSWEET6* (Medtr3g080990), *MtSWEET7* (Medtr8g099730), *MtSWEET9a* (Medtr5g092600), *MtSWEET9b* (Medtr7g007490), *MtSWEET11* (Medtr3g098930), *MtSWEET12* (Medtr8g096320), *MtSWEET13* (Medtr3g098910), *MtSWEET14* (Medtr8g096310), *MtSWEET15a* (Medtr2g007890), *MtSWEET15b* (Medtr5g067530), *MtSWEET15c* (Medtr7g405730), *MtSWEET15d* (Medtr7g405710), *MtSWEET16* (Medtr2g436310); *MtSUT4-1* (Medtr5g067470), *MtSUT4-2* (Medtr3g110880), *MtSTP13* (Medtr1g104780). Housekeeping genes for qRT-PCR: *MtPI4K* (Medtr3g091400), *MtPTB2* (Medtr3g090960), *MtUBC28* (Medtr7g116940).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. *MtSWEET11* is expressed exclusively in root hairs of infected plants and nodules.

Supplemental Figure S2. Predicted protein structure of *MtSWEET11*.

Supplemental Figure S3. *MtSWEET11* shows no transport of glucose.

Supplemental Figure S4. *MtSWEET11* expression is correlated with successful infection.

Supplemental Figure S5. *MtSWEET11*-GFP fusion expressed ectopically localizes essentially to PM and symbiosomes but no other membranes.

Supplemental Figure S6. Exon-intron structure of *MtSWEET11* in ecotype R108 and relative positions of *Tnt1* insertions.

Supplemental Figure S7. *sweet11* mutants exhibit growth characteristics similar to those of corresponding wild-type controls.

Supplemental Figure S8. BC2-F3 progenies of heterozygous BC2-F2 *sweet11* mutants show no segregation for phenotypic characteristics such as fresh weight and nitrogenase activity.

Supplemental Figure S9. Light-deprivation does not cause a gene-specific effect on growth and nitrogenase activity of *sweet11* mutants.

Supplemental Figure S10. RT-PCR with *MtSWEET11* coding sequence-specific primers on cDNA preparations from wild-type and the two mutant lines.

Supplemental Figure S11. Expression of 14 sucrose transporter genes in different organs measured by qRT-PCR relative to three housekeeping genes.

Supplemental Table S1. List of primers and sequences used in this study.

Supplemental Dataset 1. Gene IDs and coding sequences used in this study.

Supplemental Dataset 2. Genomic DNA sequences of *MtSWEET11* from ecotypes R108 and Jemalong A17.

Supplemental Dataset 3. Sugar transporters, Suc synthases, and invertases potentially relevant to the nodule function.

Supplemental Dataset 4. Relative expression of *MtSWEET11* versus other *MtSWEET* family members in different nodule zones.

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