Symbiotic nitrogen fixation occurs in nodules, specialized organs on the roots of legumes. Within nodules, host plant cells are infected with rhizobia that are encapsulated by a plant-derived membrane forming a novel organelle, the symbiosome. In *Medicago truncatula*, the symbiosome consists of the symbiosome membrane, a single rhizobium, and the soluble space between them, called the symbiosome space. The symbiosome space is enriched with plant-derived proteins, including the *M. truncatula* EARLY NODULIN8 (*MtENOD8*) protein. Here, we present evidence from green fluorescent protein (GFP) fusion experiments that the *MtENOD8* protein contains at least three symbiosome targeting domains, including its N-terminal signal peptide (SP). When ectopically expressed in nonnodulated root tissue, the *MtENOD8* SP delivers GFP to the vacuole. During the course of nodulation, there is a nodule-specific redirection of *MtENOD8*-SP-GFP from the vacuole to punctate intermediates and subsequently to symbiosomes, with redirection of *MtENOD8*-SP-GFP from the vacuole to punctate intermediates preceding intracellular rhizobial infection. Experiments with *M. truncatula* mutants having defects in rhizobial infection and symbiosome development demonstrated that the *MINIP/LATD* gene is required for redirection of the *MtENOD8*-SP-GFP from the vacuoles to punctate intermediates in nodules. Our evidence shows that *MtENOD8* has evolved redundant targeting sequences for symbiosome targeting and that intracellular localization of ectopically expressed *MtENOD8*-SP-GFP is useful as a marker for monitoring the extent of development in mutant nodules.
the apoplast, also by immunolocalization (Vanden-Bosch et al., 1994). The nodule-specific Cys-rich (NCR) peptides are thought to traverse the SymS en route to their localization in bacteroids (Van de Velde et al., 2010). Endoplasmic reticulum (ER)-localized MtDNF1 has been shown to be critical for symbiosome maturation; it encodes a putative subunit of a signal peptidase essential for NCR signal peptide (SP) cleavage (Van de Velde et al., 2010; Wang et al., 2010). Recently Hohnjec et al. (2009) demonstrated that MtNOD25’s SP was sufficient to target GFP to symbiosomes.

The MtENOD8 gene is a member of a tandemly duplicated family, of which only one copy is active in nodules (Dickstein et al., 2002). It encodes a short acyl chain esterase (Pringle and Dickstein, 2004) with an unknown role in the SymS. Its symbiosome localization makes it ideal for use as a tool to investigate mechanisms targeting proteins to this unique and important organelle-like structure. Here, we present evidence showing that MtENOD8 protein has several symbiosome-targeting signals, including its 28-amino acid SP. Additionally, we show the SP is able to direct GFP to vacuoles in uninoculated roots and to fluorescent puncta in nodules prior to and during rhizobial release from ITs, suggesting that protein trafficking to the vacuole is different in nodules than in roots.

RESULTS

Localization of MtENOD8-GFP to the SymS

In previous studies, using a combination of immunolocalization and subcellular fractionation, MtENOD8 protein was shown to be localized primarily to the SymS, with a small amount associated with the SymM (Coque et al., 2008). We tagged MtENOD8 protein at its C-terminal (CT) end with GFP, connected by a 10-Ala linker, and put the construct under MtEF1α promoter control (Fig. 1). *M. truncatula* A17 roots were transformed via *Agrobacterium rhizogenes* and the resulting transformed roots were inoculated with *Sinorhizobium meliloti* carrying a red fluorescent protein (RFP) marker (Smit et al., 2005). At 17 d postinoculation (dpi), we examined the resulting mature nodules for MtENOD8-GFP localization. As shown in Figure 2, A and B and in Supplemental Figure S1, GFP fluorescence was observed in symbiosomes, localizing with and around RFP-tagged rhizobia, demonstrating that GFP did not alter MtENOD8’s endogenous localization. We noted that in some parts of the images, the RFP and GFP signals colocalize; this is apparently caused by the close association of the SymS with the RFP-tagged elongated bacteroids. In other sections of the images, the RFP-tagged rhizobia are distinct from the surrounding GFP-labeled SymS (Fig. 2, A and B, arrows). Similar to endogenous MtENOD8, observed using immunolocalization (Coque et al., 2008), MtENOD8-GFP is found in proximal symbiosomes, but not in those in the distal zones of the nodule (Supplemental Fig. S1).

Subsequently, we expressed the *MtENOD8-GFP* construct using the strong, ubiquitous Arabidopsis (*Arabidopsis thaliana*) *EF1α* promoter (Fig. 1) and also observed GFP fluorescence in symbiosomes (Fig. 2, C and D; Supplemental Fig. S2). Only nodules were observed to contain GFP fluorescence and within them, only infected cells had a signal with GFP fluorescence localizing in and around RFP rhizobia, similar to results with *pMtENOD8* (Figs. 2, C and D and 3, A and B), even though the *AtEF1α* promoter is constitutively expressed in roots and nodules (Auriac and Timmers, 2007). Control nodules elicited on roots transformed with *pAtEF1α-GFP* showed GFP fluorescence in the cytosol and nucleus (Fig. 2, E and F; Supplemental Fig. S3). To rule out the possibility that the MtENOD8-GFP was misfolded in root and non-infected nodule cells and thus nonfluorescent, total protein was extracted from roots transformed with *pAtEF1α-MtENOD8-GFP*, blotted to membranes, and probed with anti-MtENOD8 antisera. No MtENOD8 protein was detected in nonnodulated transgenic roots, but was readily detected in extracts from wild-type control nodules (Fig. 3C). The *pAtEF1α-MtENOD8-GFP* mRNA was detected in noninoculated root tissue by semiquantitative reverse transcription (RT)-PCR using MtENOD8-specific primers (Fig. 3D). To address the remote possibility that the 27-kDa GFP tag interfered with MtENOD8 protein expression, we also fused MtENOD8 to the short *Myc* tag and attempted to express it in roots using *pAtEF1α*. Similar to MtENOD8-GFP, we failed to find evidence of MtENOD8 protein in *pAtEF1α-MtENOD8-Myc* transformed root tissue by western blot, but MtENOD8-Myc mRNA was detected by semiquantitative RT-PCR using MtENOD8-specific primers (Supplemental Fig. S4). To examine proteasome degradation as a possible cause of instability of ectopically expressed MtENOD8 fusions in roots, we tested the proteasome inhibitor, MG132 used at 50 μM for 48 h, conditions similar to those previously used in *M. truncatula* (Roudier et al., 2003), with roots transformed with *pAtEF1α-MtENOD8-Myc*. It was without effect (Supplemental Fig. S5).

Multiple Symbiosome-Targeting Domains in MtENOD8

To determine which domain of MtENOD8 is responsible for its symbiosome localization, we fused different parts of *MtENOD8* to GFP. Figure 1 shows diagrams of *MtENOD8-GFP* constructs made. The constructs were fused to the *AtEF1α* promoter, transformed into *M. truncatula* roots, nodulated with *RFP*-containing rhizobia, and observed with epifluorescence and confocal microscopy for the resulting GFP fluorescence in roots and in nodules. For these nodulation studies, we selected nodules on transgenic composite plants that were approximately the same size as nodules on wild-type plants because in our growth conditions, nodulation is asynchronous on *A. rhizogenes*-transformed composite plants (Pislariu and Dickstein, 2007). The results show that each tested construct delivered GFP fluorescence to...
the symbiosome. These constructs included those encoding the MtENOD8 SP, MtENOD8 without its SP, the N-terminal (NT) half of MtENOD8 with and without the SP, and the CT half of MtENOD8 with and without the SP (Fig. 4, A–F; Supplemental Figs. S6 and S7). In each case, similar to results shown in Figure 2, we observed a significant amount of RFP and GFP colocalization in mature nodules at the resolution possible with our confocal microscope. Thus, some of the partial MtENOD8-GFP fusions, while unambiguously localized to the symbiosome, are not unambiguously localized to the SymS, and could be in the bacteroids, similar to the NCR peptides (Van de Velde et al., 2010). However, for a MtENOD8-GFP fusion to reach a bacteroid, it must first enter the SymS.

**MtENOD8 Symbiosome-Targeting Domains: The SP and Other Potential Domains**

MtENOD8’s SP sequence is different from that of the MtNOD25-like symbiosome protein, which is conserved with that of several other symbiosome proteins and can target GFP to symbiosomes. This suggests that there are symbiosome trafficking paths yet to be discovered (Hohnjec et al., 2009). We searched the *M. truncatula* genome annotation (version 3.5) for proteins with similar SP sequences to MtENOD8’s and found few that were similar (Fig. 5A; Supplemental Table S1). Medtr1030230 is an MtENOD8 homolog found in the tandemly duplicated MtENOD8 gene cluster (Dickstein et al., 2002). None of the other similar SPs belong to proteins that are known to be expressed in symbiosomes or nodules; only one of the similar SPs belongs to a studied gene or protein: Medtr3g030400 encodes a gene corresponding to probeset Mtr.15106.1. S1 at on the Affymetrix gene chip (Benedito et al., 2008), expressed at low levels in transgenic seeds expressing a mannan synthase.

We compared the NT half of MtENOD8 to its CT half and identified two regions of sequence homology (Fig. 5B). These motifs might be potential symbiosome-targeting domains. A comparison of these motifs with other known symbiosome proteins did not return any matches.

**MtENOD8-SP-GFP Is Expressed in Root Tissue**

The experimental strategy of having all protein fusion constructs under the control of p*AtEF1a* allowed us to monitor the location of each fusion in uninoculated nodule cells and in uninoculated root tissue, cells where MtENOD8 is normally not found. As noted above, transgenic hairy roots constitutively expressing the complete MtENOD8-GFP fusion protein had neither detectable GFP fluorescence nor detectable MtENOD8 protein in nonnodulated roots (Fig. 3, A and C). Roots expressing the GFP-tagged MtENOD8 truncations were examined for GFP fluorescence in nonnodulated transgenic hairy roots. Fluorescence was only detected in the roots transformed with p*AtEF1a-MtENOD8-SP-GFP* (Fig. 6). None of the plants transformed with the other MtENOD8-GFP truncations had fluorescent root tissue in the nonnodulated state (Supplemental Fig. S8, A–E) or in uninected nodule cells (Fig. 4).

**MtENOD8-SP-GFP Localization Changes during Nodulation**

Imaging of nonnodulated transgenic roots expressing p*AtEF1a-MtENOD8-SP-GFP* showed GFP fluorescence in vacuoles in most cells (Fig. 6B). Free GFP expressed from the p*AtEF1a-GFP* control had fluorescence restricted to the nucleus and cytoplasm in most cells (Fig. 6C), indicating that the SP of MtENOD8 is sufficient for vacuolar targeting in nonnodulated roots. As can be seen in Figure 6, B and C, occasional root cells were nonfluorescent. This was not caused by the fluorescence being restricted to a different focal plane than the one shown (not shown); transformation was either incomplete or there is gene silencing present in a minority of cells.

That MtENOD8-SP-GFP is in the symbiosome in infected cells in nodules (Fig. 4A; Supplemental Fig. S6) and in vacuoles in nonnodulated roots (Fig. 6B),
show that it redistributes during nodulation. To address MtENOD8-SP-GFP’s changing localization, we examined wild-type A17 hairy roots transformed with pAtEF1α-MtENOD8-SP-GFP during a nodulation time course at 4, 6, 8, and 12 dpi. At 4 dpi, nodule bumps containing rhizobia confined to ITs were apparent, but not all nodule cells contained ITs. In root cells proximal to nodule bumps, GFP fluorescence was observed in vacuoles (Fig. 7A, arrow). Within the developing nodule cells, some cells had vacuolar MtENOD8-SP-GFP fluorescence (Fig. 7A, arrowhead), whereas the majority of cells, including cells without ITs in them, had cytosolic punctate GFP fluorescence (Fig. 7A, double arrowheads). The fluorescent foci ranged in size from 0.58 to 3.1 μm, with an average size of 1.6 ± 0.8 μm (n = 10).

At 6 dpi, very few cells in developing nodules were observed to have vacuolar GFP fluorescence, with almost all cells displaying punctate GFP fluorescence (Fig. 7, B and C) in foci ranging in size from 0.43 to 3.5 μm, with an average size of 1.3 ± 0.9 μm (n = 10). RFP-labeled rhizobia have begun to be released from ITs and are apparently in symbiosomes. Only a minority are surrounded by GFP fluorescent puncta, and few, if any, RFP rhizobia are within the GFP puncta (Fig. 7C, arrows). At this time point, there are many uninfected nodule cells with punctate GFP fluorescent foci (arrowheads). In roots at 6 dpi, GFP fluorescence was still confined to the vacuoles in the inner cortical cells proximal to the nodule (Supplemental Fig. S9), indicating that the changed distribution of MtENOD8-SP-GFP from the vacuole to punctate bodies is nodule cell specific.

Figure 2. MtENOD8-GFP localizes to the symbiosome. Transgenic roots expressing pMtENOD8-MtENOD8-GFP (A and B), pAtEF1α-MtENOD8-GFP (C and D), or pAtEF1α-GFP (E and F) were inoculated with S. meliloti expressing RFP and imaged at 17 dpi. A to D, Arrows indicate regions where MtENOD8-GFP surrounds the RFP signal, indicating SymS localization. The yellow color in sections A to D indicates overlap between GFP and RFP signals. MtENOD8-GFP fusion protein is localized to the symbiosome when it is expressed from either pMtENOD8 or pAtEF1α (compare sections A and B to C and D), while free GFP expressed from pAtEF1α is found in infected (arrowhead, sections E and F) and uninfected cells (double arrowhead, sections E and F) in the cytosol and nucleus (arrow, sections E and F). No yellow overlapping signal is observed (sections E and F). Scale bars = 25 μm (A, C, and E) and 10 μm (B, D, and F). All images are from 80-μm vibratome sections.

Figure 3. Expression of MtENOD8-GFP using a constitutive promoter shows MtENOD8-GFP only in infected nodule cells. A and B, Epifluorescent image of root (A) and nodule (B) transformed with pAtEF1α-ENOD8-GFP. No GFP fluorescence is observed in the root (A). In the nodule, GFP fluorescence is only evident in regions of the nodule corresponding to where symbiosomes are found. No GFP fluorescence is observed in the root or in the epidermal and apical regions of the nodule (B). C, Western blot of total protein extracted from nonnodulated roots transformed with pAtEF1α-MtENOD8-GFP (lanes 1 and 3) or from wild-type (A17) nodules (lanes 2 and 4). Lanes 1 and 2, Coomassie-stained blot. Lanes 3 and 4, blot destained and probed with MtENOD8-specific antiserum followed by secondary antibody. MtENOD8 protein is only observed in the nodule extract. D, Total RNA from nonnodulated roots transformed with pAtEF1α-ENOD8-GFP (lane 1) or nontransgenic wild-type (A17; lane 2) roots was analyzed by primers specific for MtENOD8 with Msc27 as positive control. MtENOD8 transcript is readily detected in transgenic roots but not in wild-type control roots. Bars = 0.5 mm in A and B. A, Whole mount; B, 80-μm vibratome section.
At 8 dpi, MtENOD8-SP-GFP fluorescent foci were abundant (Fig. 7, D and E). At this time point, the fluorescent puncta were measured to range in size from 0.58 to 6.9 μm, with an average size of 2.5 ± 2.0 μm (n = 10). More GFP fluorescent foci were localized around released RFP-fluorescing rhizobia than at 6 dpi, but similar to 6 dpi, few RFP rhizobia were in the middle of a GFP punctum (Fig. 7E, arrow). At 8 dpi, in cells at nodule apices in approximately 15% of more than 25 nodules examined in three biological replicates, GFP fluorescence was also observed along the outer regions of cells, most likely in the apoplast (Fig. 7F, arrowheads). In this region, GFP fluorescence was also observed in cell vacuoles (Fig. 7F, arrow).

At 12 dpi on A. rhizogenes-transformed roots, we observed larger, more mature nodules as well as smaller ones. These smaller nodules corresponded to developmentally younger nodules containing rhizobia released from ITs (Fig. 7, G and H). Some GFP fluorescent foci showed colocalization with RFP expressing rhizobia (Fig. 7H, arrows), but some RFP rhizobia are not within GFP puncta (Fig. 7H, arrowheads) and many GFP puncta do not contain RFP rhizobia (Fig. 7H, double arrowheads). Larger nodules at 12 dpi contain cells filled with RFP-labeled rhizobia that are beginning to elongate and are surrounded by GFP fluorescence; these appear to represent maturing symbiosomes (Fig. 7I). In these cells, there is a clear separation of GFP and RFP, indicating that the MtENOD8-SP-GFP is in the SymS, different from the elongated symbiosomes containing more mature bacteroids observed at 17 dpi (Fig. 4).

MtENOD8-SP-GFP Localization in Mutant Nodules

We were curious about pAtEF1α-MtENOD8-SP-GFP expression in mutant nodules with aberrant symbiosome development. The Mtnif1-1 mutant, defective in a signal peptidase subunit, has symbiosomes that do not mature into elongated forms (Wang et al., 2010). In Mtnip-1, defective in an apparent nitrate transporter that may have a role in nitrate or hormone signaling, the majority of nodules are blocked at release of rhizobia from ITs (Veereshingam et al., 2004; Harris and Dickstein, 2010; Yendrek et al., 2010). We transformed roots of these mutants with pAtEF1α-MtENOD8-SP-GFP, inoculated with S. meliloti harboring RFP and observed nodules at 15 dpi. We found that the Mtnif1-1 nodules displayed punctate MtENOD8-SP-GFP fluorescence, as well as punctate MtENOD8-SP-GFP.
fluorescence with RFP fluorescence in the middle (Fig. 8, A and B). In contrast, the Mtnip-1 nodules displayed GFP fluorescence mostly in vacuoles within nodule cells, with occasional areas of apoplastic staining, separate from the RFP rhizobia confined to ITs. In Mtnip-1 nodules, the GFP fluorescence did not localize to fluorescent puncta, as in Mtinf-1 nodules or in developing wild-type nodules (Fig. 8C).

**DISCUSSION**

The addition of GFP to MtENOD8’s CT end did not alter MtENOD8’s localization to the SymS in nodule cells (Figs. 1 and 2; Coque et al., 2008). Normally, the MtENOD8 protein is found highly expressed in some, but not all symbiosomes: Mature symbiosomes with elongated bacteroids contain MtENOD8, whereas younger, distal, infected nodule cells do not express it; this occurs because MtENOD8 is expressed in the proximal parts of nodules (Coque et al., 2008). When the MtENOD8-GFP fusion was regulated by the endogenous MtENOD8 promoter, expression was directed to the SymS in proximal nodule cells as expected (Fig. 2, A and B; Supplemental Fig. S1). When the MtENOD8-GFP fusion was controlled by the ubiquitous AtEF1α promoter, it was found in the SymS in all infected cells (Figs. 2, C and D and 3, A and B; Supplemental Fig. S2). When pAtEF1α-MtENOD8-GFP expression was monitored, we did not observe expression of MtENOD8-GFP in uninfected nodule cells or uninfected roots, either by fluorescence or by western blot, even though its mRNA accumulates (Figs. 2, B and C and 3; Supplemental Figs. S2 and S4). We conclude that MtENOD8-GFP is posttranscriptionally regulated, either not translated or immediately degraded in cells that do not contain symbiosomes.

To determine which domains in MtENOD8 protein are required to target GFP to symbiosomes, we fused different parts of MtENOD8 to GFP (Fig. 1). We found that MtENOD8’s SP is sufficient to target GFP to symbiosomes, similar to the case for the MtNOD25-like symbiosome protein. MtNOD25-like’s SP, also shown to be sufficient for SymS targeting, is well conserved with some symbiosome-localized proteins but is distinct from others, including MtENOD8’s SP and those of the Gly-rich protein and NCR proteins (Alunni et al., 2007; Hohnjec et al., 2009). Although MtENOD8’s SP is also able to accumulate GFP in symbiosomes, its sequence is only similar to SPs of several other proteins, one of which is an MtENOD8 homolog; none of the others have been identified as symbiosome proteins (Fig. 5).

Other parts of MtENOD8 were tested by fusion to GFP for their ability to localize GFP to symbiosomes. We found that either and both the NT and CT halves of MtENOD8 protein, with or without the SP, were each capable of targeting GFP to symbiosomes, demonstrating that at least two other cis domains of unknown sequence are capable of acting as symbiosome localization signals (Fig. 4; Supplemental Figs. S6 and S7). This suggests that there are redundant mechanisms to assure that MtENOD8 localizes to symbiosomes. Future work will dissect MtENOD8 into smaller pieces and fuse them to GFP, starting with the motifs identified in
When ectopically expressed using the *AtEF1α* promoter, MtENOD8-SP-GFP accumulates in vacuoles of root cells. In developing nodules, in cells containing ITs, with or without released rhizobia, and in cells without ITs, MtENOD8-SP-GFP localizes to fluorescent foci (Fig. 7) that average 1.8 μm in diameter. The foci are similar in size to vacuolar bodies observed in pea nodules to contain PsCYP15A (3–5 μm), measured by confocal microscopy (Vincent and Brewin, 2000). They are larger than the 100 to 300 nm multivesicular body vesicles associated with Rab5 and the 300 to 500 nm multivesicular body vesicles associated with Rab7 in nodulating *M. truncatula* roots (Limpens et al., 2009); however, these sizes were measured by electron microscopy and are thus not directly comparable. The fluorescent foci could be vesicles or vacuolar bodies overloaded with MtENOD8-SP-GFP. Early in nodule development, RFP-labeled rhizobia released from ITs are found in MtENOD8-SP-GFP expressing cells amid GFP foci, but rarely within the foci. In mature nodules, MtENOD8-SP-GFP is found in symbiosomes, surrounding the RFP rhizobia (Fig. 7). This could indicate that the MtENOD8-SP-GFP foci are capable of fusing with symbiosomes or may mean that MtENOD8-SP-GFP is redirected to symbiosomes later in nodule development. The nature of these foci is unclear, but we think

![Figure 7](image_url)

**Figure 7.** Nodulation time course of transgenic hairy roots expressing pAtEF1α-ENOD8-SP-GFP inoculated with *S. meliloti* expressing RFP. A, Four days postinoculation. Arrow points to vacuolar GFP fluorescence in root cells adjacent to the proximal region of the nodule. Single arrowhead indicates vacuolar fluorescence in interior nodule cell. Double arrowheads indicate fluorescent puncta. Arrow points to vacuolar GFP fluorescence in root cells adjacent to the proximal region of the nodule. A, Four days postinoculation. Arrow points to vacuolar GFP fluorescence in root cells adjacent to the proximal region of the nodule. Single arrowhead indicates vacuolar fluorescence in interior nodule cell. Double arrowheads indicate fluorescent puncta. B, Six days postinoculation. GFP fluorescence within nodule is primarily in fluorescent foci. C, Enlarged region of section B. Arrows indicate newly released rhizobia in symbiosomes. Arrows show GFP fluorescent puncta. Note that the GFP puncta and newly released symbiosomes are not associated and do not colocalize. D, Eight days postinoculation. E, Enlarged region of section D. Arrow indicates release of bacteria into nodule cell surrounded by fluorescent puncta. F, Eight days postinoculation. Distal region of nodule showing apoplastic fluorescence (arrowheads) as well as vacuolar localization (arrow) in the nodule epidermal cells. G, Twelve days postinoculation. H, Enlarged region of section G. Arrows point to released rhizobia colocalizing with MtENOD8-SP-GFP after release. I, Twelve days post inoculation higher magnification showing localization of MtENOD8-SP-GFP to the symbiosome. Bars = 50 μm in A, B, D, F, and G; 25 μm in C, E, and H; 10 μm in I. All images are from 80-μm vibratome sections.
it is significant that their appearance precedes rhizobial release into symbiosomes. It suggests that nodule cells may modify protein trafficking pathways in preparation for rhizobial release from ITs or it may reflect a nodule cell-type difference from root cells. Additionally, these data indicate that root vacuoles are different from nodule vacuoles.

The finding that MtENOD8-SP-GFP localizes to vacuoles in roots is unexpected, because addition of an SP to GFP is expected to target GFP to the ER, followed by movement to the apoplast (Vitale and Raikhel, 1999; Vitale and Hinz, 2005; Hunter et al., 2007). Studies performed mostly in tobacco (Nicotiana tabacum) and Arabidopsis have established that protein sorting to vacuoles requires one of two types of cis-peptide signals, each of which interacts with a specific vacuolar sorting receptor (Vitale and Hinz, 2005; Hunter et al., 2007; Rojo and Denecke, 2008; Bottanelli et al., 2011), neither of which is found in MtENOD8-SP. For retention of proteins in other intracellular compartments, positive sorting information is also thought to be required, with ER signals also well characterized. However, soluble Golgi or endosomal compartment proteins have not been reported; thus their retention signals are not known (Rojo and Denecke, 2008). It was shown previously that overexpression of a secreted form of GFP results in its targeting to puncta identified as part of the vacuolar pathway. This was interpreted as the secreted GFP form containing a cryptic vacuolar-targeting signal (Zheng et al., 2005). Thus, MtENOD8-SP-GFP’s vacuolar localization in roots and fluorescent puncta in developing nodule cells could be a result of the SP containing a cryptic vacuolar signal. Alternatively it could be an artifact of...
ectopic expression (Foresti et al., 2008; Moore and Murphy, 2009). We do note that a small fraction (approximately 15%) of nascent nodules expressing MtENOD8-SP-GFP contain apical cells that have apparent apoplastic GFP fluorescence (Fig. 7E). In these few cells, MtENOD8-SP-GFP could have overloaded the cellular trafficking system that takes it to the vacuole, leading to its secretion. Alternatively, secretion could have been a result of the expected trafficking: MtENOD8-SP-GFP undergoing cleavage of its SP, yielding 10-Ala GFP that is exported, as has been observed for cytosolic or foreign proteins with an attached SP (Vitale and Raikhel, 1999). Future experiments will investigate SPs from other symbiosome proteins in our system. They will include colocalization of MtENOD8-SP-GFP and other SP fusions with membrane and soluble protein markers that traffic to defined intracellular compartments, but it is important to note that such markers are only just beginning to be used in *M. truncatula* (Pumplin et al., 2012).

Recently a nodule-specific signal peptidase subunit localizing to the ER, Mtdnf1, was identified in *M. truncatula* that is required for the nitrogen-fixing symbiosis (Wang et al., 2010). Mtdnf1 is responsible for cleaving SPs from the NCR proteins required for bacteroid maturation (Van de Velde et al., 2010). Wang et al. (2010) proposed MtENOD8’s SP could be a potential substrate for Mtdnf1. If that were the case, one would expect MtENOD8-SP-GFP to localize differently in *Mtdnf1*-1 nodules compared with wild type. We observed a similar localization for MtENOD8-SP-GFP in *Mtdnf1*-1 nodules as for wild type in the middle stages of nodulation (compare Fig. 7I with Fig. 8, A and B), suggesting that Mtdnf1 is not involved in processing MtENOD8’s SP.

Alternative pathways are possible to symbiosomes, as suggested previously (Catalano et al., 2004; Hohnjec et al., 2009; Limpens et al., 2009): MtENOD8-SP-GFP could be translated on cytosolic ribosomes, with the SP directing it to symbiosomes in nodule cells. We note that in plants there is no known pathway for proteins to localize in vacuoles after translation on cytosolic ribosomes (Marty, 1999; Vitale and Raikhel, 1999; Vitale and Hinz, 2005; Rojo and Denecke, 2008). In yeast (*Saccharomyces cerevisiae*) however, autophagy-like mechanisms for cytoplasm to vacuole targeting have been described (Baba et al., 1997). Another alternative is that MtENOD8-SP-GFP could be secreted to the apoplast and endocyted to the vacuole or symbiosomes. In the latter case, the observed fluorescent foci in young nodules might be compartments of the endocytosis pathway.

There are earlier reports of symbiosome-localized proteins that also localize to other cellular compartments. Peanut nodule lectin is in vacuoles and the extracellular matrix in uninfected nodule parenchyma cells, whereas in infected cells, it is in symbiosomes (VandenBosch et al., 1994). Pea Cys protease PsCYP15A localizes to vacuolar bodies, cytoplasmic vesicles, vacuoles, and symbiosomes in nodules (Vincent and Brewin, 2000). PsNLEC-1 is in vacuoles and symbiosomes (Dahiya et al., 1997). As suggested above, we cannot rule out that MtENOD8-SP-GFP vacuolar localization in roots could be a GFP-tagging-induced artifact, delivered to the vacuole after entering the ER lumen but not subsequently degraded. If so, it might be expected to continually sort there, which it does not. Either way, our results suggest that the initial route for MtENOD8-SP-GFP symbiosome targeting has common elements with vacuolar targeting, as do several other symbiosome proteins.

For whole-protein MtENOD8 trafficking to symbiosomes, we suggest that it is translated on ER ribosomes and subsequently sorted to symbiosomes. Multiple domains in MtENOD8’s SP and NT and CT halves ensure that it localizes to symbiosomes.

Figure 9 presents a hypothesis for MtENOD8-SP-GFP trafficking. We propose that MtENOD8-SP-GFP is translated on ER ribosomes and sorted from the ER or Golgi bodies to vacuoles in root cells or symbiosomes in infected nodule cells. We suggest that MtENOD8’s SP is not cleaved in the ER and serves as an address label to symbiosomes in nodule cells, similar to an alternative proposed by Hohnjec et al. (2009) for the MtNOD25-like SP.

Ectopically expressed MtENOD8-SP-GFP localization is useful as a marker during nodulation. *Mtnip*-1 nodules have numerous rhizobia in ITs that only rarely release into symbiosomes and were observed to have unusually large vacuoles (Veereshlingam et al., 2004). *Mtnip*-1 has defects in MtNIP/LATD (Yendrek et al., 2010), a member of the NRT1 (PTR) family of nitrate and small peptide transporters, that also includes a nitrate sensor able to transport auxin (Ho et al., 2009; Krouk et al., 2010; Gojon et al., 2011). How MtNIP/LATD functions during nodulation is still an open question. We found that MtENOD8-SP-GFP fluorescence was mostly vacuolar in *Mtnip*-1 nodules (Fig. 8), showing that MtNIP/LATD is required for MtENOD8-SP-GFP’s relocalization during nodulation (Fig. 9). This could imply that *Mtnip*-1 nodule cells’ vacuoles are more like root vacuoles than nodule vacuoles and that *Mtnip*-1 nodules, in addition to being defective in rhizobial release from ITs, may be unable to modify protein trafficking pathways in preparation for rhizobial release or may be unable to differentiate correctly from root to nodule cells.

**MATERIALS AND METHODS**

**Plants, Growth Conditions, and Rhizobial Strains**

*Meditago truncatula* A17 and nodulation mutants were grown in aeroponic chambers, starved of nitrogen for 5 d, and inoculated with *Sinorhizobium meliloti* containing an RFP reporter gene (Smit et al., 2005), as described previously (Veereshlingam et al., 2004).

**Protein Extraction and Western Blotting**

Total protein extraction was done by harvesting A17 and mutant nodules at 15 dpi and freezing them in liquid nitrogen. Frozen tissue was ground to a powder under liquid nitrogen with a mortar and pestle, resuspended in...
2 μL of protein extraction buffer containing 100 μL Tris pH 8.0, 100 μL NaCl, 3 μL EDTA, 2% SDS, 5% β-mercaptoethanol with 1 μL of protease inhibitor cocktail (Sigma-Aldrich) per 100 μL of protein extraction buffer, per mg of tissue, vigorously vortexed, and heated at 95°C for 5 min. Samples were clarified by centrifugation at 16,000 g for 15 min. The supernatant was collected and protein concentration was determined using a DC protein assay kit (Bio-Rad).

For western blots, 30 μg total protein per lane was resolved on a 10% acrylamide gel, transferred to a polyvinylidene fluoride membrane, stained with Coomassie Blue, and scanned for an image. Stained membranes were cleared overnight in Tris-buffered saline (TBS) buffer containing 0.3% Tween 20 (TBS plus Tween 20). After being cleared of Coomassie Blue, membranes were blocked in blocking buffer (TBS plus Tween 20 containing 5% nonfat dry milk) at 25°C for 1 h. Membranes were immersed in blocking buffer containing 1:4,000 anti-ENOD8 antisera (Dickstein et al., 2002) for 1 h, rinsed for 5 min five times in TBS, incubated in blocking buffer containing 1:4,000 goat anti-rabbit IgG alkaline phosphatase conjugated antibody (Bethyl Laboratories), and rinsed for 5 min five times in TBS. Membranes were then equilibrated in 100 μL Tris pH 9.5 and developed with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (Fishar Scientific).

RT-PCR

Total RNA from root tissue was extracted using an Ambion PureLink RNA mini kit (Applied Biosystems). Two micrograms of RNA was used to synthesize cDNA using the Promega GoTaq 2-Step RT-PCR system (Promega). Primers specific for MtENOD8 were used to verify MtENOD8 expression with Misc7 used as a constitutive control (Allison et al., 1993). All primer sequences are in Supplemental Table S2.

Constructs

M. truncatula ENOD8 cDNA. GenBank accession AF064775 (Li et al., 1999). MtENOD8 was cloned into pRD22 (Pislariu and Dickstein, 2007) using Ncol/Nhe restriction sites engineered into MtENOD8 creating pRD082. See Supplemental Table S1 for all primer sequences. pAEIFa-SmGFP (Auriac and Timmers, 2007) was used as a template for AEIFa, which was cloned into pRD042 using BamHI/NcoI restriction sites engineered into AEIFa creating pHM021. An in-frame c-Myc tag (EQKLISEEDL) was engineered into pMH021 using self-complementary oligonucleotides containing Nhel and BstXI overhangs into the Nhel/BstXI sites within pMH021 creating pMH027. A BstXI/BstXI digested fragment of pHM027 containing AEIFa-MtENOD8-c-Myc was cloned into pCAMBIA2301 creating pHM021M. A 3.047-bp fragment containing AEIFa-MtENOD8-c-Myc and the Tnos terminator from pHM023 was cloned into pRedRooll (Limpens et al., 2004) using HindIII/KpnI sites that were engineered into the 5' and 3' sites, respectively, creating pHM019. Vectors containing MtENOD8-GFP fusions were created by cloning GFP from pCY44 (Yendrek et al., 2010) in frame with MtENOD8 in pHM021 using Nhel and BstXI engineered sites into GFP creating pHM030. A linker encoding 10 Ala was engineered into the forward primer to serve as a spacer between MtENOD8 and GFP. For MtENOD8-GFP fusion truncations, all MtENOD8 variants were engineered containing Nhel/Ncol sites at their 5' and 3' ends, respectively, and cloned into pHM030, resulting in in-frame GFP fusions. For MtENOD8-SP, an 84-bp product corresponding to the first 28 amino acids of MtENOD8 was amplified and cloned, creating pHM311. A 1.059-bp fragment corresponding to NT-CT lacking the MtENOD8-SP was amplified and cloned, creating pHM313. The first 573 bp of MtENOD8 were chosen as the NT half (including the SP) and the last 570 bp as the CT half. For SP-NT the first 573 bp of MtENOD8 was cloned creating pHM329. For NT only, a 489-bp fragment lacking the first 28 amino acid coding region was cloned, creating pHM325. For CT only, the 570-bp 3' end of MtENOD8 was cloned, creating pHM327. To create SP-CT a fusion PCR strategy was used. The 84-bp coding region corresponding to SP coding sequence was amplified using a reverse primer containing a 5' end complementary to the first 18 bp of CT. The entire CT was amplified using a forward primer that contained a 5' end complementary to the last 18 bp at the SP's 3' end. These PCR products were purified and mixed in a PCR reaction containing the SP and CT hybrid products and a SP forward primer and a CT reverse primer such that only SP-CT fusion product would be amplified. The fused SP-CT product was cloned, creating pHM331. All constructs were cloned as BamHI/BstXI plasmid constructs into pCAMBIA2301.

For free GFP control studies, GFP was engineered with Ncol and Nhel sites and cloned into pMH021, resulting in pMS020. The reverse primer contained sequences encoding the 10-Ala residue linker into the NT of GFP so that this construct could be used as a free GFP control and for NT GFP fusion proteins. A BamHI/BstEI fragment of pMS023 was cloned into pCAMBIA2301, resulting in pMS026. The integrity of all plasmids was verified by sequencing and restriction digests.

Generation of Transgenic Composite Plants with Transformed Hairy Roots

Binary vector constructs were transformed into the Agrobacterium rhizobium strain ARqual. Transgenic hairy roots were generated as previously described (Pislariu and Dickstein, 2007). After 19 to 24 d on kanamycin selection, composite plants were transferred to aeroponic chambers and nodulated or analyzed for protein or RNA content as described above.

Fluorescence and Confocal Microscopy

Transgenic hairy roots were screened for GFP fluorescence by epifluorescence on a Nikon e600 compound microscope equipped with a DXM1200F camera (Nikon Instruments Inc.) and e-cite 120 Fluor system (Exfo Life Sciences Division). For confocal microscopy of MtENOD8-SP transformed roots, nonnodulated transgenic hairy roots, whole-root sections were mounted in water, covered with a coverslip, and imaged using a Leica TCS SP2 AOPS (Leica Microsystems) equipped with a 63× HCX Plan Apo objective. For confocal microscopy of nodule sections, 80-μm-thick sections were obtained from fresh tissue using a 1000 Plus Vibratome (Vibratome). Sections were mounted in water and imaged using a Zeiss 200M inverted optical microscope (Carl Zeiss) attached to a CSU-10 Yokogawa spinning confocal scanner (Yokogawa) and Hamamatsu electron multiplier CCD camera (Hamamatsu). Resulting images were processed using ImageJ software (http://rsweb.nih.gov/ij/Abramoff et al., 2004).

SP Analysis

SPs were identified from the first 35 amino acids of putative proteins in the M. truncatula genome annotation, version 3.5 (downloaded from http://www.medicago.hapmap.org/downloads_genome/Mt35/Mt35v3_annotation_files.tar.gz), for multiple sequence alignment, which was visualized using the Boxshade program (Rice et al., 2000). The T-Coffee server (Notredame et al., 2000) was used by using the SignalP program (Bendtsen et al., 2004; Frank and Sippl, 2008). Medicagohapmap.org/downloads_genome/Mt3.5/Mt3.5v3_annotation_files.tar.gz), M. truncatula genome annotation, version 3.5 (downloaded from http://www.medicago.hapmap.org/downloads_genome/Mt35/Mt35v3_annotation_files.tar.gz), by using the SignalP program (Bendtsen et al., 2004; Frank and Sippl, 2008). Putative SPs were compared with MtENOD8's SP using EMBOSS Needle (Rice et al., 2000). The T-Coffee server (Notredame et al., 2000) was used for multiple sequence alignment, which was visualized using the Boxshade program (Rice et al., 2000).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF064775 and AF465407.

Supplemental Data

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

Supplemental Figure S1. Expression of pMtENOD8-MtENOD8-GFP in transgenic hairy roots inoculated with S. meliloti expressing RFP.

Supplemental Figure S2. Expression of pAEIFa-MtENOD8-GFP in transgenic hairy roots inoculated with S. meliloti expressing RFP.

Supplemental Figure S3. Expression of pAEIFa-GFP in transgenic hairy roots inoculated with S. meliloti expressing RFP.

Supplemental Figure S4. Western immunoblot and RNA analysis of nonnodulated roots expressing pAEIFa-MtENOD8-Myc.
Supplemental Figure S5. Analysis of MG132-treated transgenic roots expressing pMENOD8-MtENOD8-Myc for MtENOD8 mRNA and protein.

Supplemental Figure S6. Localization of MtENOD8-GFP truncations to the symbiosome.

Supplemental Figure S7. Localization of MtENOD8-GFP truncations to the symbiosome.

Supplemental Figure S8. Epifluorescent images of transgenic roots expressing MtENOD8-GFP truncations.

Supplemental Figure S9. MtENOD8-SP-GFP root vacuolar localization at 6 dpi.

Supplemental Table S1. Predicted M. truncatula proteins with putative signal peptides.

Supplemental Table S2. Sequences of PCR primers used in this work.

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Meckfessel et al.


Supplemental Figure 1. Expression of pMtENOD8-MtENOD8-GFP in transgenic hairy roots inoculated with S. meliloti expressing RFP. A, D, G, MtENOD8-GFP fluorescence localizes to the symbiosome. B, E, H, RFP fluorescence. C, F, I, Merged images. Arrows indicate symbiosomes. Panels A-F show MtENOD8-GFP localized to symbiosomes. Panels G-I show lower magnification of a root nodule. MtENOD8 is expressed in zone III; no MtENOD8-GFP fluorescence is observed in zone II. Arrowhead indicates cell in zone II where MtENOD8 is not expressed. A-C and G-I bars = 25 µm, D-F bars = 10 µm. All images from 80 µm vibratome sections.
Supplemental Figure 2. Expression of pAtEF1α-MtENOD8-GFP in transgenic hairy roots inoculated with S. meliloti expressing RFP. A and D, MtENOD8-GFP fluorescence to the symbiosome. B and E, RFP fluorescence. C and F, Merged images. Arrows indicate symbiosomes. Yellow color indicates overlap between the GFP and RFP signals showing symbiosome localization of MtENOD8-GFP under pAtEF1α expression. A-C bars = 25 µm, D-F bars = 10 µm. All images from 80 µm vibratome sections.
Supplemental Figure 3. Expression of \textit{pAtEF1a-GFP} in transgenic hairy roots inoculated with \textit{S. meliloti} expressing \textit{RFP}. A and D, Free GFP fluorescence to the cytosol of infected and uninfected cells and the nucleus. B and E, RFP fluorescence. C and F, Merged images. Arrow indicates a nucleus. Arrowheads indicate cytoplasmic GFP fluorescence in infected cells. Double arrowheads indicate GFP fluorescence in uninfected cells. Note the lack of yellow color indicating no co-localization between GFP and RFP signals. A-C bars = 25 \(\mu\)m, D-F bars = 10 \(\mu\)m. All images from 80 \(\mu\)m vibratome sections.
Supplemental Figure 4. Western immunoblot and RNA analysis of non-nodulated roots expressing pAtEF1α-MtENOD8-Myc. A, Western blot of total protein; left, Coomassie stained membrane; right, blot destained and probed with MtENOD8-specific antisera followed by secondary antibody. No protein is evident in non-nodulated roots expressing MtENOD8-Myc but is readily detected in nodules. B, RT-PCR of total RNA using primers specific for MtENOD8, with primers for Msc27 as a positive control. MtENOD8 transcript is detected in roots expressing MtENOD8-Myc as well as nodules.
Supplemental Figure 5. Analysis of MG132 treated transgenic roots expressing pAtEF1α-MtENOD8-Myc for MtENOD8 mRNA and protein. A, Total RNA was extracted and amplified using primers specific for MtENOD8 and Msc27. MtENOD8 mRNA is present in transgenic roots expressing pAtEF1α-MtENOD8-Myc treated and untreated with MG132. No mRNA is evident in transgenic roots transformed with an empty vector. B, Western blot of total protein extracted from roots expressing pAtEF1α-MtENOD8-Myc and empty vector stained with Coomassie (left) and probed with anti-MtENOD8 sera (right). No MtENOD8 protein is detected in any of the samples from transformed roots, but is readily detected in nodule protein extract.
Supplemental Figure 6. Localization of MtENOD8-GFP truncations to the symbiosome. Transgenic roots were inoculated with *S. meliloti* expressing *RFP*. A-C, MtENOD8-SP-GFP. D-F, MtENOD8-NT-CT-GFP. G-H, MtENOD8-SP-NT-GFP. A,D,G, GFP fluorescence from MtENOD8 fusions. B,E,H, RFP fluorescence from *S. meliloti*. C,F,I, Merged images. Arrows indicate positions where non-overlap of GFP and RFP were observed. Arrowheads indicate ITs. The yellow color found in all MtENOD8-GFP truncation merged images indicates co-localization between the GFP signal from the MtENOD8 fusion proteins and the *S. meliloti* expressing RFP. Note the extensive co-localization of GFP and RFP signals, except for RFP fluorescing ITs (arrowheads), indicating that each MtENOD8 truncation fused to GFP localizes to symbiosomes. Bars = 25μm. All images are from 80μm vibratome sections.
Supplemental Figure 7. Localization of MtENOD8-GFP truncations to the symbiosome. Transgenic roots were inoculated with *S. meliloti* expressing RFP. A-C, MtENOD8-NT-GFP. D-F, MtENOD8-SP-CT-GFP. G-H, MtENOD8-CT-GFP. A,D,G, GFP fluorescence from MtENOD8 fusions. B,E,H, RFP fluorescence from *S. meliloti*. C,F,I, Merged images. Arrows indicate positions where non-overlap of GFP and RFP were observed. Arrowheads indicate ITs. The yellow color found in all MtENOD8-GFP truncation merged images indicates co-localization between the GFP signal from the MtENOD8 fusion proteins and the *S. meliloti* expressing RFP. Note the extensive co-localization of GFP and RFP signals, except for RFP fluorescing ITs (arrowheads), indicating that each MtENOD8 truncation fused to GFP localizes to symbiosomes. Bars = 25μm. All images are from 80μm vibratome sections.
Supplemental Figure 8. Epifluorescent images of transgenic roots expressing *MtENOD8-GFP* truncations. Red arrows indicate untransformed root; yellow arrows indicate emerged transgenic hairy roots. Roots are transformed with the following constructs: A, *MtENOD8-NT-CT-GFP*; B, *MtENOD8-SP-NT*; C, *MtENOD8-NT*; D, *MtENOD8-SP-CT*; E, *MtENOD8-CT*. No fluorescence is detected in any of the transgenic roots. Bars = 0.5mm; all images are from whole mounts.
Supplemental Figure 9. MtENOD8-SP-GFP root vacuolar localization at 6 dpi. In root cells proximal to the developing nodule, MtENOD8-SP-GFP continues to localize to the vacuole (arrows) and does not redistribute to fluorescent puncta as in the developing nodules. Bar = 50 µm. Image of 80 µm vibratome section.