The Signal Peptide of the *Medicago truncatula* Modular Nodulin MtNOD25 Operates as an Address Label for the Specific Targeting of Proteins to Nitrogen-Fixing Symbiosomes

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The nodule-specific *MtNOD25* gene of the model legume *Medicago truncatula* encodes a modular nodulin composed of different repetitive modules flanked by distinct N- and C-termini. Although similarities are low with respect to all repetitive modules, both the N-terminal signal peptide (SP) and the C-terminus are highly conserved in modular nodulins from different legumes. On the cellular level, *MtNOD25* is only transcribed in the infected cells of root nodules, and this activation is mediated by a 299-bp minimal promoter containing an organ-specific element. By expressing mGFP6 translational fusions in transgenic nodules, we show that MtNOD25 proteins are exclusively translocated to the symbiosomes of infected cells. This specific targeting only requires an N-terminal MtNOD25 SP that is highly conserved across a family of legume-specific symbiosome proteins. Our finding sheds light on one possible mechanism for the delivery of host proteins to the symbiosomes of infected root nodule cells and, in addition, defines a short molecular address label of only 24 amino acids whose N-terminal presence is sufficient to translocate proteins across the peribacteroid membrane.

Additional keywords: alternative splicing, confocal laser-scanning microscopy, mRFP.

The root nodule symbiosis between legumes and rhizobial prokaryotes represents the most intensively studied nitrogen-fixing plant-microbe interaction (Crespi and Galvez 2000; Schultze and Kondorosi 1998). This extremely host-specific symbiosis is initiated in the root cortex (Geurts and Bisseling 2002) after the perception of rhizobial Nod factors through LysM-domain-containing receptor kinases (Cullimore and Dénarié 2003; Oldroyd and Downie 2006). Subsequent signal transduction triggers the expression of plant genes located downstream of the Nod-factor perception pathway, ultimately resulting in root nodule organogenesis.

Root nodules are colonized by rhizobia through tubular infection threads (Brewin 1991). Subsequent to an endocytotic uptake of rhizobia at the infection thread tips, cells from the inner nodule zones house nitrogen-fixing microsymbionts in transient organelle-like structures termed symbiosomes (Parniske 2000). Here, rhizobia differentiate into bacteroids capable of carrying out the conversion of dinitrogen to ammonia. To drive bacteroid energy metabolism, legumes supply dicarboxylic acids to the endosymbionts via specific dicarboxylic acid transporters located in a specialized symbiotic interface, the largely plant-derived peribacteroid membrane (PBM) (Day et al. 2001). In exchange, ammonia is released through PBM channels (Tyerman et al. 1995) to the plant cytoplasm, where it is incorporated into glutamine by glutamine synthetase (Carvalho et al. 2003). In addition to the nitrogen-fixing bacteroid, the PBM surrounds the acidic peribacteroid space (PBS) (Day et al. 2001; Udvardi and Day 1997). Ultimately, it is the coordinated exchange of carbon for nitrogen metabolites across the PBS and PBM that enables legumes to colonize nitrogen-depleted soils, a property exploited in sustainable agriculture (Graham et al. 2004).

An early goal in research on nitrogen-fixing symbioses was the identification of plant genes that initiate and support nodule development. Classical methods used to address this question were differential display and subtractive hybridization techniques. Although these approaches identified dozens of nodulation-related genes in different legumes (Gamas et al. 1996; Pawlowski 1997; Perlick and Pühler 1993), recent genome-enabled studies widened our view of symbiosis-related gene expression by identifying several hundred nodule-activated genes (Colebatch et al. 2004; El Yahyaoui et al. 2004; Fedorova et al. 2002; Journet et al. 2002; Küster et al. 2004). Nodule-specific proteins were traditionally designated nodulins (van Kammen 1984) and were discriminated into early and late nodulins according to their time of synthesis during nodule development. For most nodulin genes, a biological function remains to be assigned.

In a study on nodulation-related genes of broad bean (*Vicia faba* L.), 19 early and late nodulin genes were identified by differential hybridization (Perlick and Pühler 1993). Among those, the *VfNOD28/32* gene encoded modular nodulins composed of different repetitive modules flanked by distinct N- and C-termini (Küster et al. 1994, 1996), reminiscent of the *MsNOD25* gene encoding nodulin-25 of *Medicago sativa*

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(Kiss et al. 1990a). Interestingly, the number and order of repetitive modules was species specific, indicating that the function of these proteins was related to the individual modules (Küster et al. 1999). In *Vicia* spp., genes specifying modular nodulins were exclusively expressed in the nitrogen-fixing zone, around the onset of nitrogen fixation (Küster et al. 1996, 1999). Expression in nonsymbiotic tissues was not reported for *VfNOD28/32* or for homologous genes from *V. hirsuta* (Küster et al. 1999), *M. sativa* (Kiss et al. 1990a), or *Galega orientalis* (Kajjalainen et al. 2002).

Fig. 1. Sequence properties of modular nodulins from *Medicago truncatula* cvs. Jemalong and R108. **A**, Comparison of nodulin-25 proteins encoded by the *MtNOD25* tentative consensus sequences (TCs) TC113614, TC119488, and TC115445. Modules identified are indicated by arrows. White arrow, N module; black arrow, repeat 1 module; dark-gray arrow, repeat 2 module; light-gray arrow, repeat 3 module; striped arrow, C module. Alterations in the deduced amino acid sequence of TC115445 (*M. truncatula* cv. R108) as opposed to TC113614 and TC119488 (both *M. truncatula* cv. Jemalong) are marked in bold, and predicted signal peptides (SPs) are underlined. The relation of modules to exons of the *MtNOD25* gene isolated from cv. Jemalong is indicated, including two pseudoeons (PE1 and PE2) that correspond to modules found only in TC115445, probably due to differences in the exon/intron splice sites in the two cultivars. **B**, Modules of nodulin-25 proteins. Residues identical in at least 50% of the sequences are marked in bold and the SP predicted by Signal P (Emanuelsson et al. 2007) is underlined. **C**, Comparison of the C-termini of modular nodulins from *M. truncatula* (MtNOD25), *M. sativa* (MsNOD25) (Kiss et al. 1990a), *Vicia faba* (VfNOD32a) (Küster et al. 1994), and *Galega orientalis* (GOCA-04) (Kajjalainen et al. 2002).

In recent years, proteomics experiments revealed that some 200 legume proteins are specifically translocated to either the PBS or PBM (Catalano et al. 2004; Saalbach et al. 2002; Wienkoop and Saalbach 2003). To ensure the correct targeting of these proteins, three major pathways were proposed by Catalano and associates (2004), two of them relying on N-terminal signal peptides (SP) that mediate either cotranslational or posttranslational protein translocation. In their study, Catalano and associates (2004) reported that *M. truncatula* modular nodulins (nodulin-25, encoded by the *MtNOD25* gene) are associated with the inner side of the PBM, adding evidence to an earlier hypothesis by Kiss and associates (1990a) that these proteins are located at the symbiotic interface.

Recently, Liu and associates (2006) identified the calmodulin-like peribacteroid proteins MtCaML1-6. The corresponding genes were located in the vicinity of the nodulin genes *MtN22* (Gamas et al. 1996) and *MtNOD25*, leading to the hypothesis that a conserved nodule-specific 5' sequence encoding an SP was translocated to *MtCaML1-6* via exon shuffling (Liu et al. 2006). The authors also found that homologous 5' regions existed in the *M. sativa* nodulin gene *Nms-32/34*, and in four *Lotus japonicus* and two *Lupinus luteus* transcript sequences (Ganter et al. 1998; Liu et al. 2006; Swiderski et al. 2000).

Due to their proposed peribacteroid localization, an elucidation of the subcellular translocation of modular nodulins was of interest in order to improve the understanding of protein targeting to symbiosomes. Together with an in situ expression study of *MtNOD25* in root nodules, we here present the subcellular localization of MtNOD25-mGFP6 translational fusions in the infected cells. Our results indicate that, similar to the MtCaML proteins (Liu et al. 2006), nodulin-25 is specifically translocated to the symbiosomes. Moreover, we demonstrate that the N-terminal SP of MtNOD25 found to be conserved across different symbiosome proteins is indeed sufficient for this subcellular targeting. This finding on the one hand sheds light on one possible mechanism for protein targeting in the infected cells of root nodules and on the other hand provides the first experimental evidence for a short molecular-address label capable of specifically directing legume proteins into the vicinity of nitrogen-fixing bacteroids.

RESULTS AND DISCUSSION

Properties of the *MtNOD25* gene encoding modular nodulins of *M. truncatula*.

Three different *MtNOD25* transcript sequences encoding modular nodulins are listed in the Dana Farber Cancer Institute (DFCI) *M. truncatula* Gene Index 9. Similar to modular nodulins from *V. faba*, *V. hirsuta*, and *M. sativa* (Küster et al. 1994, 1999; Kiss et al. 1990a), MtNOD25 proteins consist of different repetitive modules flanked by distinct N- and C-termini (Fig. 1A and B). The altered number of repetitive modules in *MtNOD25* cDNAs (Fig. 1A) indicates the existence of splice variants, reminiscent of the situation in *Vicia* spp. (Küster et al. 1999). Whereas only limited homologies exist between repetitive sequences of modular nodulins from different legumes (data not shown), the C-terminal modules are highly similar (Fig. 1C), indicating a conserved function. Based on SignalP (Emanuelsson et al. 2007), the N-terminus of MtNOD25 contains an SP with a cleavage site between amino acids 24 and 25 (Fig. 1A and B).

Nodule expressed sequence tags (EST) encoding modular nodulins were found in *Medicago*, *Galega*, and *Vicia* spp., all belonging to the galeoid legumes (Doyle and Luckow 2003) but not in the phaseoloid legumes *Glycine max* and *Lotus japonicus*. To infer putative functions for modular nodulins, we

performed sequence- and motif-based homology searches against the current releases of GenBank, Swissprot (Bairoch et al. 2004), Uniprot (Boutet et al. 2007), and Interpro (Quevillon et al. 2005); however, these searches identified no significantly homologous sequences from nonlegumes. This indicates that modular nodulins are legume-specific proteins (Graham et al. 2004) and, although 5' gene sequences encoding MtNOD25-like N-terminal SP are present in legumes forming determinate nodules (Liu et al. 2006), are characteristic of those nodules where bacteroids undergo terminal differentiation. In these nodules, other legume-specific proteins (e.g., cysteine-cluster and glycine-rich proteins) were proposed to play a role during the terminal differentiation of bacteroids (Alunni et al. 2007; Frühling et al. 2000; Mergaert et al. 2003).

The *MtNOD25* gene is located in a symbiosis island.

In the current *M. truncatula* genome release 2.0, an incomplete *MtNOD25* sequence is present on BAC Mth2-124L21. To clone the complete *MtNOD25* gene, overlapping BACs (Nam et al. 1999) were identified using cDNA probes. Two of these BACs contained a 10.5-kb *EcoRI* fragment that was also observed in genomic Southern hybridizations (Supplementary Fig. S1). In addition to the complete sequence of this fragment (AJ277858, Supplementary Fig. S2), the terminal sequences of four *MtNOD25* BACs (AJ311512 to AJ311519) were obtained. BAC Mth2-124L21 partially matched the sequence of the 10.5-kb *EcoRI* fragment, indicating that *MtNOD25* maps at ≈ 34 centimorgans on chromosome III (Thoquet et al. 2002). To the left of *MtNOD25*, this contig contained the nodule-specific gene *MtN22* (Gamas et al. 1996) and the *MtCaML6* gene (Fedorova et al. 2002) encoding a nodule-specific calmodulin-like protein. The identification of *MtN22* and *MtCaML6* next to *MtNOD25* confirms the report of Liu et al. (2006) that *MtNOD25* is located downstream of *MtN22* and a family of six *MtCaML* genes, in a genomic region discussed as a symbiosis island (Zhu et al. 2003).

The general exon/intron structure of *MtNOD25* is similar to that of the *MsNOD25* gene (Végh et al. 1990) (Supplementary Fig. S3). Interestingly, a repetitive module missing in several *MtNOD25* transcripts (Fig. 1A) corresponds to exon E7. This finding confirms that alternative splicing can generate different *MtNOD25* variants. In addition to the 13 *MtNOD25* exons covered by EST, a pseud exon (PE1, position 7,428 to 7,481) has a typical *MtNOD25* exon sequence but is probably not included in *M. truncatula* cv. Jemalong *MtNOD25* transcripts due to an altered 5' splice site.

The *MtNOD25* gene is exclusively expressed in the infected cells of *M. truncatula* nodules.

MtNOD25 gene expression was determined by real-time reverse-transcription polymerase chain reaction (RT-PCR) in young and mature root nodules, arbuscular mycorrhizal (AM) roots, uninfected roots, leaves, flowers, and pods. These experiments revealed exclusive *MtNOD25* expression in nodules, whereas the gene was not induced in AM roots or nonsymbiotic tissues (data not shown). The strictly nodule-specific expression is supported by the fact that more than 100 *MtNOD25* EST from the DFCI *M. truncatula* Gene Index 9 (Quackenbush et al. 2001) were exclusively derived from nodules or nodulated roots. In addition, the *MtNOD25* promoter sequence was similar to the promoter sequence of the *VfNOD28/32* gene that was specifically active in the central region of root nodules (Fig. 2) (Küster et al. 1999).

To monitor the accumulation of *MtNOD25* transcripts within nodule tissues, in situ hybridizations were carried out using *M. truncatula* root nodules at 25 days postinoculation (dpi) with *Sinorhizobium meliloti*. In longitudinal sections

(Fig. 3A and B), enhanced *MtNOD25* transcript levels occurred in the infected cells of the nitrogen-fixing zone, whereas control hybridizations with a sense probe did not exhibit comparable staining (data not shown).

Preceding the *MtNOD25* gene, a 1,462-bp promoter region contained a motif resembling the organ-specific element (OSE) of leghemoglobin gene promoters. We narrowed down the *MtNOD25* promoter sequences mediating nodule-specific expression by the use of promoter-*gusAint* fusions expressed in transgenic *M. truncatula* nodules. Histochemical analyses showed that deletions up to position -1,106 did not affect strong and specific expression in the infected cells of root nodules (Fig. 3C and D), similar to the activity of the *MsNOD25* promoter (Kiss et al. 1990b). Deletions down to position -299 (Fig. 3E and F) had no qualitative effect on gene expression, although the expression intensity was somewhat reduced. Furthermore, deletion -299 of the *MtNOD25* promoter lost its exclusive expression in the infected cells, because it displayed a residual activity in the nodule vasculature (Fig. 3F). A further deletion up to position -101 removed the 5' portion of the OSE and abolished promoter activity completely (data not shown), indicating that the major control elements driving gene expression in the infected cells reside between positions -101 and -299. This is in line with a high sequence identity between modular nodulin gene promoters from *V. faba* and *M. truncatula* in this area (Fig. 2) and matches the observation that *MtCaML1* and *MtCaML2* sequences are similar to *MtNOD25* promoters in this region as well (Liu et al. 2006). Nevertheless, upstream sequences contribute to expression intensity and to an exclusive *MtNOD25* expression in the infected cells. This resembles the situation in soybean nodulin genes, where OSE require the presence of upstream activating sequences to drive gene expression to the infected cells of root nodules (Stougaard et al. 1990).

MtNOD25-mGFP6 fusions are targeted to the symbiosomes.

To investigate the subcellular localization of *MtNOD25* in the infected cells of root nodules, different translational fusions to the *mGFP6* reporter gene were constructed (Fig. 4) and their expression was monitored in transgenic *M. truncatula* nodules (Fig. 5). Because the *Cauliflower mosaic virus* (CaMV) 35S promoter is primarily expressed in the uninfected cells of root nodules and, thus, is not well suited for studying the localization of translational fusions in the infected cells of central nodule tissues (Auriac and Timmers 2007), we have chosen the

<i>VfNOD28/32</i>	AATATTAGAAAGGTCATTGTAAAGTAGTTATGTACA--CCGAAATTAACCTAAAGTATT	-292
<i>MtNOD25</i>	TGATATGCCACATTCATTGTGAAGTGTAAAGAGATACCAAGTAACTTAAATATT	-287
<i>VfNOD28/32</i>	ACAAACTTATAAACTTCTAAAGCTACATTTGTTTAAATT--GAAATAAAT-TAG	-235
<i>MtNOD25</i>	GAAACGTATGAACATTTACAGCTAGATTTCAATTTTAAATTTAGAAAGAGAACCTAT	-227
<i>VfNOD28/32</i>	GCCTTTGAAAGACCAAAATTTGAGAACTCCAAATAAATACTATATAAATGTGTGTGCATGT	-175
<i>MtNOD25</i>	GCCTTCGAAT-ATATAAATTTTAAATTTCCAAATAAATAAATAAATAAATATAT-----	-175
<i>VfNOD28/32</i>	TAAATGTTTAAATATTAACTAGATATATCTCAAAATTTGCAATGCCACATTTT	-115
<i>MtNOD25</i>	TGCTTTTAA--ATATTAAATATGATCAATTTCTTTAGAAAGTTATATGTCACATTTGT	-117
<i>VfNOD28/32</i>	TC-TTTTAAATTTAAGATGACCTCTCCCAATAAGTAGCCATATTTGGGCGCAT	-56
<i>MtNOD25</i>	TAATTTAACCTAAT-TTAAATGACCTCTGGCCATAAATAAGTCATCTTTAACCCAT	-58
<i>VfNOD28/32</i>	ATATAGAGCCTA--TATTGTTTATGAGCATTCGATTCGCAATCTCCAAAGTAATAATG	+03
<i>MtNOD25</i>	ATATAGAGACTACATATTATTATCAAGCATTCGATTTTCAATCTCCAAATAGCAAAATG	+03

Fig. 2. Comparison of the immediate upstream promoter regions of *VfNOD28/32* and *MtNOD25*. Identical positions are marked by asterisks. A region resembling organ-specific elements of leghemoglobin and other nodulin gene promoters is shown inverted, whereas putative TATA-boxes are marked in bold. The transcriptional start site of *VfNOD28/32* (Küster et al. 1999) and the most 5' residues of *MtNOD25* cDNAs (TC113614) are underlined, whereas the ATG start codons are additionally shown in italics. An *HpaI* site defining the extent of the -299 deletion of the *MtNOD25* promoter is underlined.

native *MtNOD25* promoter (−1,072/−5) to drive expression of *MtNOD25-mGFP6* fusions (Fig. 4). An inspection of transgenic *pN25-N25*–green fluorescent protein (*GFP*) nodules colonized by an *S. meliloti* strain expressing a constitutive m-red fluorescent protein (*RFP*) (Smit et al. 2005) revealed co-

Fig. 3. Cellular expression of the *MtNOD25* gene in *Medicago truncatula* root nodules. **A** and **B**, Localization of *MtNOD25* transcripts in *M. truncatula* root nodules. **A**, Longitudinal cross section of a mature *M. truncatula* root nodule hybridized against an *MtNOD25* antisense target. Highest expression is found in the infected cells throughout the nitrogen-fixing zone III, whereas control hybridizations with a sense probe did not exhibit any comparable staining (*data not shown*). **B**, Magnification of the area boxed in **A**. *MtNOD25* transcripts are detected only in the infected cells. **C** through **F**, Activity of p*MtNOD25*–*gusAint* fusions in sections of transgenic *M. truncatula* root nodules: **C**, −1,462/−4; **D**, −1,106/−4; **E**, −436/−4; and **F**, −299/−4 promoter regions. Except for the −299/−4 p*MtNOD25*–*gusAint* fusion displaying weak activity in the nodule vasculature, strong and specific promoter activity was detected in the infected cells of the central nodule tissue. Abbreviations: in, infected cells; un, uninfected cells.

localization of mGFP and mRFP in the infected cells of root nodules (Fig. 5A), with no leakage of the fusion proteins to the uninfected cells being evident (Fig. 5A and B). Subcellular GFP fluorescence was observed in distinct circular and sickle-like structures surrounding the bacteroids (Fig. 5C), whereas the vacuole, nucleus, cell membrane, and cell wall of the infected cells did not display GFP fluorescence (Fig. 5B). Together, the analysis of GFP fluorescence in transgenic root nodules indicates an exclusive localization of *MtNOD25*–mGFP6 fusion proteins in the infected cells (Fig. 5A through C), in accordance with the expression pattern of the *MtNOD25* promoter (Fig. 3). On the subcellular level, our data demonstrate that *MtNOD25*–mGFP6 fusions are efficiently and specifically targeted to the symbiosomes in the infected cells of root nodules. Together with the MtCaML protein family described by Liu and associates (2006) and the ENOD8 protein encoding a nodule-specific esterase (Coque et al. 2008), modular nodulins are among the few known legume proteins that are approved to pass through the PBM to accumulate in the PBS, thus contributing to a specific protein composition of the symbiosome.

The *MtNOD25* signal peptide targets mGFP6 proteins to the symbiosome.

The N-terminal module of *MtNOD25* largely consists of an SP (Fig. 1) with a proposed cleavage site between amino acids 24 and 25 (SignalP-score 0.98) (Emanuelsson et al. 2007). On the structural level, this SP consists of a characteristic basic n-region and a classical hydrophobic h-region as well as a typical polar c-region, and its amino acid sequence conforms to the −3/−1 rule defined by von Heijne (1986). Together, these properties result in an SP probability of 0.99 and a signal anchor probability of only 0.01 according to SignalP (Emanuelsson et al. 2007), allowing the conclusion that the *MtNOD25* SP is most probably cleaved and does not function to anchor *MtNOD25* in the PBM.

Significant homologies of the N-terminal *MtNOD25* module exist not only to N-termini of six *M. truncatula* genes encoding nodule-specific calmodulin-like proteins (MtCaML), the *M. truncatula* nodulin gene *MtN22*, and the *M. sativa* nodulin gene *Nms-32/34*, but also to *Lupinus luteus* and *Lotus japonicus* EST (Ganter et al. 1998; Liu et al. 2006; Swiderski et al. 2000). These similarities in all cases are confined to the N-terminal region belonging to the predicted SP (Fig. 6A), with sequence identities or similarities declining immediately after the SP cleavage site proposed by SignalP (Emanuelsson et al. 2007). This finding allowed us to define a characteristic *MtNOD25*–like SP motif visualized in Figure 6B. Similar to nodulin-25 (discussed above), all other proteins containing an

Fig. 4. Fusions of defined regions of the *MtNOD25* protein to mGFP6. The complete *MtNOD25* coding region (pN25–N25–GFP), the *MtNOD25* N-module (pN25–SP34–GFP) region, and the *MtNOD25* (pN25–SP24–GFP) region were fused to the *mGFP6* reporter gene. Expression of all fusions is mediated by the *MtNOD25* promoter fragment from position −1,072 to −5 (relative to the start codon). A dotted line denotes an amino acid linker. Abbreviations: SP, signal peptide-encoding *MtNOD25* region; GFP, coding region for the green fluorescent protein; pN25, promoter of the *MtNOD25* gene.

N-terminal MtNOD25-like SP have a high SP probability and a low signal anchor probability according to SignalP (data not shown) (Emanuelsson et al. 2007). The remaining protein sequences of MtCaML1-6, MtN22, and all other proteins containing an MtNOD25-like SP motif (Fig. 6A) displayed no similarities to MtNOD25, and no SP from any other known protein displayed a comparable similarity to the MtNOD25 SP; therefore, it is likely that the SP itself contains sufficient information for protein targeting to symbiosomes.

To test whether the MtNOD25 SP is able to accumulate mGFP6 proteins in the symbiosome as does the full-size MtNOD25mGFP construct presented above, we fused two regions deriving from the N-terminal MtNOD25 module to the mGFP6 reporter gene (Fig. 4). Including the start codon, the encoded N-terminal peptides consisted of the first 34 and 24 amino acids of MtNOD25, respectively, followed by a GGRSPGGS linker (discussed below) and the mGFP6 coding region, resulting in constructs *pN25-SP34-GFP* and *pN25-SP24-GFP*, respectively. Because the sequence of the SP was not altered, these fusions were all predicted to be cleaved

between amino acids 24 and 25 (SignalP) (Emanuelsson et al. 2007).

When analyzing nodule cells colonized by an *S. meliloti* strain expressing a constitutive mRFP protein (Smit et al. 2005), we observed the same pattern of GFP expression as detailed above for the full-size *pN25-N25-GFP* fusion, regardless of the presence of 34 (Fig. 5D) or only 24 amino acids preceding the mGFP6 sequence (Fig. 5E and G through I, respectively). Again, GFP fluorescence closely and completely surrounded the space occupied by the bacteroids (Fig. 5D and E) in the infected cells, visualized via co-localization with red fluorescent *S. meliloti* (Fig. 5I). This GFP pattern excluded a translocation to the bacteroids and indicated an accumulation of MtNOD25-mGFP6 fusion proteins in the PBS. Most importantly, GFP fluorescence was observed throughout the apparent lumen of the peribacteroid space, with no visible space between GFP fluorescence and mRFP-labeled bacteroids. This observation confirms that the N-terminal presence of an original 24-amino-acid MtNOD25 SP is sufficient to direct specific GFP fluorescence to the symbiosomes of infected root nodule cells.

Fig. 5. Confocal imaging of MtNOD25-mGFP6 fluorescence in transgenic root nodules. **A** through **C**, Green fluorescent protein (GFP) fluorescence in a transgenic nodule expressing a *pN25-N25-GFP* translational fusion. **A**, Merged picture of GFP and m-red fluorescent protein (RFP) of a longitudinal nodule section colonized with *S. meliloti* strain 2011-mRFP. GFP is detected in the infected cells of the central nodule tissue. Red fluorescence is detected in the prefixing-zone, interzone II-III, infection threads, and infected cells of the nitrogen-fixing zone III (Vasse et al. 1990). Note that the yellow coloration in the infected cells occurs only when the signal intensity of both fluorophores is located at the same scan layer. No co-localization is visible in the prefixing zone. **B**, GFP localization in the central nodule tissue showing exclusive GFP detection within the infected cells. **C**, Close-up of a single infected cell showing circular and sickle-like GFP signals. **D**, Localization of GFP fluorescence in an infected cell expressing the *pN25-SP34-GFP* fusion. **E**, Localization of GFP fluorescence in an infected cell expressing the *pN25-SP24-GFP* fusion. **F**, Localization of GFP fluorescence in an infected cell expressing the *pN25-SP34-GFP* fusion upon colonization with the *fix⁻* mutant strain 1021 *nifH::Tn5*. **G** through **I**, Localization of GFP and mRFP in an infected cell expressing the *pN25-SP24-GFP* fusion upon colonization with the red fluorescent *S. meliloti* strain Sm2011-mRFP. **G**, GFP localization. **H**, mRFP localization of the same layer shown in **G**. The mRFP signal uncovers the presence and density of bacteroids within the infected cell. **I**, Merged picture of the mGFP6 image (**G**) and the mRFP image (**H**). The bacteroids are closely surrounded by the GFP signal. Image **B** is a superimposed image based on three optical sections. All other images represent single optical sections. Scale bars are indicated.

To prove whether the NOD25 protein localization in the symbiosome is depended on active nitrogen fixation, we used the *S. meliloti* mutant Sm1021 *nifH::Tn5* to nodulate *M. truncatula*. In fix⁺ root nodules, GFP fluorescence was restricted to a group of cells immediately below the interzone II-III region (data not shown) and, here, the symbiosome localization of GFP was comparable with the situation in root nodules colonized by wild-type rhizobia (Fig. 5F). The absence of fluorescence in regions of root nodules with more mature infected cells (data not shown) indicates that the MtNOD25 promoter is inactive in the absence of symbiotic nitrogen fixation, adding evidence that the regulation and function of nodulin-25 is tightly connected to the nitrogen fixation process.

Possible routes for the symbiosome targeting of MtNOD25 proteins.

Searches of the available *M. truncatula* genome sequence and the DFCI *M. truncatula* Gene Index did not result in the identification of proteins containing MtNOD25-like signal peptides in addition to MtCaML1-6 and MtN22 (Fig. 6), indicating that the mode of symbiosome targeting by MtNOD25-like signal peptides cannot account for the ~200 proteins that have been identified in this compartment by proteomics

(Catalano et al. 2004; Saalbach et al. 2002; Wienkoop and Saalbach 2003). Thus, it is possible that a special targeting mechanism is used by MtNOD25-like SP. Theoretically, symbiosome targeting can occur post-translationally, subsequent to protein synthesis on cytoplasmic ribosomes, or co-translationally, via synthesis into the lumen of the rER and subsequent vesicle transport to symbiosomes (pathway I and II/III, respectively; according to Catalano and associates [2004]). Assuming that the MtNOD25 SP is indeed cleaved by a signal peptidase upon translocation into the rER, the subsequent specific accumulation of mGFP6 in the symbiosome would require that a targeting via rER or Golgi complexes operates independently of the SP, possibly via specific glycosylation or another decoration of the remaining amino acid sequence. In case of the fusion of only the 24 amino acids of the N-terminal MtNOD25 SP to *mGFP6*, such a modification could only reside in the GFP sequence itself; however, cytoplasmic GFP proteins are not known to be translocated across endomembranes without the presence of an N-terminal SP. Thus, it is possible that the N-terminal MtNOD25 SP is cleaved later during co-translational PBS targeting and, thus, could still function as an N-terminal targeting signal during the passage via the rER and the Golgi complex.

Because such a delayed cleavage of SP would be unusual (Nothwehr and Gordon 1990), it appears more likely that MtNOD25-like SP operate post-translationally. One example for a post-translational SP targeting in plants is given by Chen and associates (2004). Using transgenic tobacco and rice protoplasts, the authors demonstrated that the rice α -amylase (α Amy3) is only transferred into both chloroplasts and extracellular compartments when it contains its SP, whereas α Amy3 Δ SP, missing the SP, remains in the cytoplasm. The authors could show that the SP alone was sufficient to mediate a specific translocation of GFP into chloroplasts (Chen et al. 2004). Similar to this process, symbiosome translocation of MtNOD25 apparently exclusively requires its N-terminal SP. Thus, it could be assumed that this SP directly enters the symbiosome membrane instead of the rER and, upon this, would directly be cleaved at the site of cargo destination. Catalano and associates (2004) had identified MtNOD25 proteins as components of the PBM fraction but, as stated by the authors, this co-purification might have resulted from an association of MtNOD25 with the inner PBM layer and, thus, does not exclude a PBS localization. Although the spatial resolution in fluorescence microscopy is limited, our subcellular localizations—in addition to the molecular properties of the MtNOD25 SP detailed above—are in favor of a release of MtNOD25 in the lumen of the PBS.

Together, our results elucidate one of several molecular mechanism for protein targeting to nitrogen-fixing symbiosomes by defining a short, 24-amino-acid address label. All other modules of MtNOD25, including the C-terminus that is particularly conserved across modular nodulins from different legumes (Fig. 1), are not involved in symbiosome targeting and, thus, are probably linked to the so far elusive function of MtNOD25 proteins.

Conclusions and perspectives.

In addition to the genes discussed here, large families of glycine-rich protein (GRP) and cysteine-cluster protein (NCR) genes are exclusively present in nodules where terminal bacteroid differentiation occurs, and it was proposed that the NCR and GRP proteins might be involved in controlling this differentiation process (Alunni et al. 2007; Mergaert et al. 2003). Due to their specific symbiosome localization, a possible function of MtNOD25 proteins might also be related to the fact that bacteroids differentiate over time in *M. truncatula* root

A

MtNod25*	MVYSN-SYMFGLGLGVFVLLSSHVLA-YN
MsNod25	MVYSN-TYMLLGLGVFVLLSSHVLA-YN
VfNOD28/32	MAYSN-SYMLFLLGVSLIISSQVLA-YN
MtCaML1*	MGYSN-SYLFGLNLFLVFLSSQALA-DI
MtCaML2*	MAYSK-SYLFGLNLFLVFLSFEVLA-DI
MtCaML5*	MVYSK-SYLFGLNLFLVFLSSQALA-DI
MtCaML3	MAYSK-SYFLSINLFVFLSSQVLA-DI
MtCaML6	MPYSK-SYFLCLVFFVFIIFSLVAA-DI
MtCaML4	MDHSK-LYLFGLGFLYVISSQILA-DS
MtN22	MANSK-VY--VFVGLFVVLSSQVLA-QS
nms32/34	SK-AY--VFVGLSVILSSQVLA-QS
L1DD4A9	MVNSK-EFLFFGLLLFVFLSFQVLA-HD
L1DD2T15	MVNSK-EFLFFGLLLFVFLSFLQVLA-HD
LjTC12171	ACSKSYDLYHGLGLFVLIISPVFA-HV
LjTC9989	MASSKS--QCLGLGVFVILFSQVFS-HT
LjTC9398	ASSKSYGLYLGLGLFVILFSQVFA-HT
LjTC8059	MASSKTSDLYLGLGLFVILFSQVFA-HT

B

Fig. 6. Comparison of signal peptides from different nodule-specific proteins. **A**, Comparison of the N-terminal signal peptides (SPs) of the modular nodulins MtNOD25, MsNOD25 (Kiss et al. 1990a), and VfNOD28/32 (Küster et al. 1994) to the SPs of the *Medicago truncatula* MtCaML1-6 proteins (MtCaML1, DFCI Mt Gene Index TC134855; MtCaML2, TC114830; MtCaML5, TC125585; MtCaML3, TC114830; MtCaML6, TC118427; MtCaML4, TC114792) (Liu et al. 2006), *M. truncatula* nodulin MtN22 (Gamas et al. 1996), *M. sativa* nodulin nms32/34 (Ganter et al. 1998), *Lotus japonicus* nodulins encoded by cDNAs TC12171, TC9989, TC9398, and TC8059 (Liu et al. 2006), as well as the lupin nodulins L1DD4A9 and L1DD2T15 (Swiderski et al. 2000). Prediction was done using SignalP (Emanuelsson et al. 2007), and the first 24 amino acids up to the predicted cleavage site are shown. The most frequent amino acids are shown in bold. Proteins located in the symbiosome by proteomics (Catalano et al. 2004) or GFP fusions (this work, Liu et al. 2006) are marked by asterisks. **B**, Sequence logo of the alignment shown in A (Crooks et al. 2004).

nodules. Interestingly, the sequences of MtNOD25-like SP are clearly different from those of the GRP and NCR proteins as well as the ENOD8 esterase (Alunni et al. 2007; Coque et al. 2008; Mergaert et al. 2003). Thus, it remains to be elucidated whether SP with different primary sequences can also mediate symbiosome targeting on their own or whether other targeting signals are involved. Finally, our results not only contribute to the identification of molecular mechanisms facilitating symbiosome targeting but also define a short address label that can be used as a molecular tool to target proteins or peptides to the symbiosomes in order to engineer the protein composition of the PBS or to deliver peptides that influence the metabolism of nitrogen-fixing bacteroids or the signal exchange between both symbionts.

MATERIALS AND METHODS

Plants, bacterial strains, libraries, recombinant DNA techniques, and DNA sequencing.

Seed of wild type *M. truncatula* Jemalong A17 were surface sterilized, scarified, and vernalized as described by Hohnjec and associates (2003). After germination, plants were grown in washed and sterilized clay granulate (Seramis, Masterfoods GmbH, Verden, Germany) using *S. meliloti* 1021 (Meade and Signer 1977) or *Glomus intraradices* (Premier Tech Biotechnologies, Riviere-du-Loup, Canada) for nodulation and mycorrhization, respectively, as described previously (Hohnjec et al. 2003).

To visualize *S. meliloti* inside root nodules, strain Sm2011-mRFP expressing a monomeric red fluorescent protein (Smit et al. 2005) was used. Plants harvested for in situ hybridizations were nodulated with *S. meliloti* RCR2011 pXLGD4 (Journet et al. 2002), and nodules were collected at 25 dpi. Strain Sm1021 *nifH*::Tn5 (provided by A. Becker, Institute for Genome Research and Systems Biology, Bielefeld University, Germany) was used to induce fix⁻ root nodules.

M. truncatula cv. Jemalong J5 (Gamas et al. 1996) and *M. truncatula* cv. R108 (Györgyey et al. 2000) nodule cDNA libraries were used to isolate full-length cDNAs. Filters representing a genomic BAC library of *M. truncatula* cv. Jemalong A17 (Nam et al. 1999) were obtained from D. Cook (University of California–Davis, Davis, U.S.A.). Screening of cDNA and genomic libraries was performed using standard protocols (Sambrook et al. 1989).

All in vitro DNA manipulations and cloning experiments were carried out in *Escherichia coli* XL1Blue (Bullock et al. 1987) using pK18 (Pridmore 1987) or pBluescript (Stratagene Europe, Amsterdam, The Netherlands) plasmids. Plasmid DNA was prepared using the Plasmid Mini Kit (Qiagen, Hilden, Germany), and in vitro manipulations of DNA were performed using standard protocols (Sambrook et al. 1989). DNA sequencing was performed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Darmstadt, Germany).

Identification of full-length MtNOD25 transcript sequences.

Using an *MtNOD25* RT-PCR product from *M. truncatula* nodule RNA, full-length *MtNOD25* transcript sequences were isolated from root nodule cDNA libraries of *M. truncatula* cv. R108 (Györgyey et al. 2000) and *M. truncatula* cv. Jemalong J5 (Gamas et al. 1996). Sequence analysis revealed that cDNA *MtNOD25-3* (GenBank accession AJ308588) from *M. truncatula* cv. R108 as well as cDNAs *MtNOD25-213*, *-12*, and *-25* (AJ277859, AJ277860, and AJ277861) from *M. truncatula* cv. Jemalong J5 represented complete or near-complete *MtNOD25* coding regions.

In the DFCI *Medicago truncatula* Gene Index 9, three tentative consensus sequences (TC) cover complete or near-com-

plete *MtNOD25* sequences (Fig. 1A). Specifically, cDNA *MtNOD25-3* is part of TC115445, cDNAs *MtNOD25-25* and *-213* are assembled in TC113614, and cDNA *MtNOD25-12* contributes to TC119488. Whereas TC113614 and TC119488 are based only on EST from *M. truncatula* cv. Jemalong, TC115455 was assembled from *M. truncatula* cv. R108 EST. Additional *MtNOD25* sequences were chimeric or contained only partial *MtNOD25* coding regions that were identical to the three TC described above. Because they did not reveal any additional information, they were not considered further.

Southern hybridization.

Southern hybridizations were carried out using ≈ 10 μ g of genomic *M. truncatula* cv. Jemalong A17 DNA or ≈ 1 μ g of BAC DNA digested with appropriate restriction enzymes, respectively. Digests were separated on 1% (wt/vol) agarose gels and vacuum blotted onto nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany). Hybridizations were carried out at 42°C in DIG Easy Hyb solution (Roche Diagnostics GmbH) using digoxigenin (DIG)-labeled probes (PCR DIG Probe Synthesis Kit; Roche Diagnostics GmbH). Washes were carried out at 65°C in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) for 5 min and in 0.5 $\times$ SSC and 0.1% (wt/vol) SDS for 15 min. Detections were carried out using the DIG Luminescent Detection Kit (Roche Diagnostics GmbH).

Real-time RT-PCR experiments.

Total RNA was isolated using the RNeasy Plant Mini Kit in conjunction with DNaseI treatment (Qiagen). Real-time RT-PCR experiments were performed as reported previously (Hohnjec et al. 2005) using primers specific for *MtNOD25* (TGTTGAATCACTTAAGTTAGG and TAAGCAAATGGT TTATTCAG) and *MtTef-1alpha* (Hohnjec et al. 2003). Real-time RT-PCR products were scored as detectable if they were amplified after less than 40 PCR cycles.

In situ hybridizations.

DIG-labeled sense and antisense riboprobes of *MtNOD25* were obtained from a linearized plasmid containing a full-length *MtNOD25* cDNA using the DIG RNA labeling Kit (Spe6/T7; Roche Diagnostics GmbH). Subsequent to DIG-labeling, alkaline hydrolysis delivered labeled RNA fragments of ≈ 150 bases. For embedding in paraffin, nodules were fixed in 4% (wt/vol) paraformaldehyde and 0.1% (vol/vol) Triton X-100 in phosphate-buffered saline following the protocol of Schaarschmidt and associates (2006). Embedded nodules were cut in median longitudinal sections of 8 μ m using a rotary microtome (HM355; Microm International, Walldorf, Germany). Sections were mounted onto slides coated with poly-L-lysine (Sigma-Aldrich), and the hybridization procedure was performed as described by Schaarschmidt and associates 2006. Micrographs were taken using a Zeiss Axioplan microscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with a video camera (Fujix Digital Camera HC-300Z; Fuji Photo Film, Tokyo) and were processed via Photoshop 7.0 (Adobe Systems, San Jose, CA, U.S.A.).

Constructing gusAint fusions of promoter regions and histological analysis of transgenic nodules.

The *MtNOD25* promoter was PCR amplified from a pK18 plasmid carrying a 10,581-bp genomic *MtNOD25* *EcoRI* fragment (GenBank accession AJ277858), using the M13 reverse primer and a primer that introduced a *HindIII* site immediately following position -8 of the *MtNOD25* promoter. Parts of the amplified native promoter regions were cloned as *EcoRI/HindIII* ($-1,462/-4$), *NsiI/HindIII* ($-1,106/-4$), *StuI/HindIII* ($-436/-4$),

HpaI/HindIII (–299/–4), and *SwaI/HindIII* (–101/–4) fragments (positions relative to the start codon of the *MtNOD25* gene) in front of the *gusAint* gene in plasmid pGUS-INT (Küster et al. 1995), making use of the *HindIII* site next to the *gusAint* start codon. All *gusAint* fusions were cloned as *SpeI* fragments into the *XbaI* site of plasmid pIV2 (Küster et al. 1995). All pIV2 derivatives were electroporated into *Agrobacterium rhizogenes* ARqual (Quandt et al. 1993), where they integrated into the T_L DNA by a single crossover. *A. rhizogenes* strains were used to generate transgenic roots on *M. truncatula* cv. Jemalong A17 according to Vieweg and associates (2004). Between 2 and 3 weeks postinoculation with *S. meliloti* 1021, transgenic nodules were sectioned and stained for β-glucuronidase activity as reported by Hohnjec and associates (2003). Stained sections were examined by light microscopy and documented with an Olympus C-4040Z (Olympus, Hamburg, Germany) digital camera.

Construction of MtNOD25-mGFP6 fusions and expression in planta.

We used a derivative of the p35S-GFP vector (GenBank accession number U28417) (Clontech Laboratories, Palo Alto, CA, U.S.A.). An *mGFP6* (Haseloff 1999; Haseloff et al. 1997) coding sequence was PCR amplified (AAAGGATCCATGCA TAAAGGAGAAGAAGCTTTTCACTGG and AAAGAGCTCT CACCCATCCTTTTGTATAGTTCATCCAT) and introduced as *BamHI/SacI* fragment into p35S-GFP to replace the original GFP, resulting in plasmid p35S-mGFP6 (Holger Densow, Biometra GmbH, Göttingen, Germany).

Furthermore, we introduced an *XbaI/BamHI* (TCTAGAGG TACCAGATCTCCCGGGGGATCC) linker between the original *XbaI* and *BamHI* sites of p35S-mGFP6 to expand the mcs in front of the *mGFP6* coding region in p35S-mGFP6, resulting in vector p35S-OLI-mGFP6.

For an expression under native conditions, gene-specific primers extended with suitable *SphI* and *KpnI* restriction sites (AAAGCATGCGAATGATGATGAGGAAGAAA and AAAG GTACCTTATTGGAGAAATGAAAATG, respectively), were used to amplify an *MtNOD25* promoter fragment from position –1,072 to –5 (relative to the start codon). Subsequently, we replaced the CaMV p35S promoter in p35S-OLI-mGFP6 by introducing the *MtNOD25* promoter via the *SphI/KpnI* restriction sites to create vector pN25-OLI-mGFP6.

Gene-specific primers extended with suitable *KpnI* or *BglII* restriction sites (AAAGGTACCAAAAATGGTTTATTCAAAT TCA and AAAAGATCTTCTCCTTCACTGGCAATATCATT, respectively) were used to amplify the complete *MtNOD25* coding region (TC113614). During PCR, the three adenosine bases preceding the *MtNOD25* start codon were conserved and the *MtNOD25* reading frame was completely amplified up to and including the codon preceding the stop codon.

The *MtNOD25* N-module derivatives SP34 and SP24 were also amplified, introducing suitable *KpnI* and *BglII* restriction sites, respectively (AAAGGTACCAAAAATGGTTTATTCAA ATTCA, AAAAGATCTTCTCCTCCATGGATAGAGGGATCAG CACG and AAAGGTACCAAAAATGGTTTATTCAAATTCA, AAAAGATCTTCTCCTCCAGCCAAAACATGAGAAGAGAG), resulting in PCR products of 102 and 72 bp, respectively, of the native *MtNOD25* sequence, commencing with the start codon. Again, three adenosine bases were retained in front of the ATG. To provide a sufficiently long amino acid linker between *MtNOD25* and *mGFP6* sequences, two codons for the amino acid glycine were introduced during PCR amplification. Together with the cloning site, this linker inserted a GGRSPGGS peptide linker between *MtNOD25* and *mGFP6*. All cDNA derivatives were cloned in front of the *mGFP6* coding region in plasmid pN25-OLI-mGFP6, resulting in the

translational fusion *pN25-N25-GFP*, *pN25-SP34-GFP*, and *pN25-SP24-GFP*. The integrity of these fusions was verified by restriction analysis and DNA sequencing.

The *pN25-N25-GFP*, *pN25-SP34-GFP*, and *pN25-SP24-GFP* fusions were cloned as *EcoRI/HindIII* fragments into the binary plasmid pBIN19 (Bevan 1984). The binary vector derivatives were electroporated into *A. rhizogenes* ARqual (Quandt et al. 1993) and the resulting strains were used to generate transgenic roots on *M. truncatula* cv. Jemalong A17 according to Quandt and associates (1993) on solid media containing Kan₄₀ for selection of transgenic hairy roots. After 3 weeks, plants were transferred into open pots filled with clay granulate (Seramis, Masterfoods GmbH, Verden, Germany) and nodulated with the microsymbiont as described above.

Transgenic *M. truncatula* roots were analyzed by confocal laser-scanning microscopy (CLSM). Semi-thin sections (80 μm) of root nodules were obtained as described by Hohnjec and associates (2003) using a Leica VT 1000S vibratome (Leica, Heidelberg, Germany). In vivo visualization of mGFP6 and mRFP (Smit et al. 2005) fluorophores was performed with a Leica TCS SP2 AOBs CLSM system equipped with an argon and a helium laser, respectively (emission at 488 and 543 nm) (Leica, Heidelberg, Germany). Laser scanning was performed using settings for excitation and emission wavelengths at 488 and 500 to 535 nm and 543 and 600 to 630 nm, respectively. Image processing was carried out with the Leica confocal software lite (version 2.61; Leica). For Figure 4B, a series of three optical sections, each with a 0.2-μm interval on the z-axis, was collected and superimposed to create an extended depth-of-focus image. To differentiate true GFP signals (peaking at ≈514 nm) from autofluorescence, images were routinely verified by lambda scans.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

DFCI *Medicago truncatula* Gene Index: compbio.dfci.harvard.edu/tgi
Medicago genome sequencing project: www.medicago.org/genome
 National Center for Biotechnology Information website:
www.ncbi.nlm.nih.gov
 University of California-Berkeley WebLogo3 software:
weblogo.berkeley.edu/logo.cgi