FUNCTION OF THE *ENOD8* GENE IN NODULES OF *Medicago truncatula*

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To elaborate on the function(s) of the ENOD8 gene in the nodules of M. truncatula, several different experimental approaches were used. A census of the ENOD8 genes was first completed indicating that only ENOD8.1 (nt10554-12564 of GenBank AF463407) is highly expressed in nodule tissues. A maltose binding protein-ENOD8 fusion protein was made with an E. coli recombinant system. A variety of biochemical assays were undertaken with the MBP-ENOD8 recombinant protein expressed in E. coli, which did not yield the esterase activity observed for ENOD8 protein nodule fractions purified from M. sativa, tested on general esterase substrates, \( \alpha \)-naphthyl acetate, and \( p \)-nitrophenylacetate. Attempts were also made to express ENOD8 in a Pichia pastoris system; no ENOD8 protein could be detected from Pichia pastoris strains which were transformed with the ENOD8 expression cassette. Additionally, it was shown that the ENOD8 protein can be recombinantly synthesized by Nicotiana benthamiana in a soluble form, which could be tested for activity toward esterase substrates, bearing resemblance to nodule compounds, such as the Nod factor. Transcription localization studies using an ENOD8 promoter gusA fusion indicated that ENOD8 is expressed in the bacteroid-invaded zone of the nodule. The ENOD8 protein was also detected in that same zone by immunolocalization. Confocal immunomicroscopy with an affinity-purified anti-ENOD8 oligopeptide antibody showed that the ENOD8 protein localizes at the interface between the plant and the bacteroid-
differentiated rhizobia, in the symbiosome membrane or symbiosome space. This suggests a possible link between ENOD8 protein and bacteroid differentiation, nitrogen fixation, or plant defense. These possible functions for ENOD8 could be tested with an ENOD8-RNAi transgenic line devoid of detectable ENOD8 proteins.
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by

Laurent Coque
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<tr>
<td>ALA</td>
<td>δ-aminolevulinic acid</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>Bar⁻</td>
<td>Bacterial release inability</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<td>BCIP</td>
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<td>Keyhole limpet hemocyanin</td>
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<td>LB</td>
<td>Luria Bertani</td>
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<td>LSLB</td>
<td>Low salt Luria Bertani</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
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<td>Description</td>
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<td>MDH</td>
<td>Minimal dextrose with histidine</td>
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<td>Minimal methanol with histidine.</td>
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<td>NBT</td>
<td>Nitro blue tetrazolium</td>
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<td>NCBI</td>
<td>National center for biotechnology information</td>
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<tr>
<td>NGS</td>
<td>Normal goat serum</td>
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<td>National institutes of health</td>
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<td>PCR</td>
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<td>Post-transcriptional gene silencing</td>
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<td>Tris-acetate EDTA</td>
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<tr>
<td>TBE</td>
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<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TBSV</td>
<td>Tomato bushy stunt virus</td>
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<tr>
<td>TEMED</td>
<td>N, N, N’, N’-tetramethylethlenediamine</td>
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<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>TY</td>
<td>Trypton yeast extract</td>
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</tbody>
</table>
X-Gal   5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc  5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
YPD    Yeast extract peptone dextrose medium
YPDS   Yeast extract peptone dextrose sorbitol medium
YNB    Yeast nitrogen base

Genes

ENOD    Early nodulin gene
AOX1    Alcohol oxidase 1
exo     Exopolysaccharide biosynthesis or regulation genes
hemA    ALA synthesis
ndv     Locus involved in nodules development
Nod     Rhizobium nodulation genes

Medicago truncatula Lines

A17     M. truncatula genotype A17
2HA     M. truncatula genotype 2HA

Bacterial Strains

Agrobacterium tumefaciens AGL1 and C58C1
Agrobacterium rhizogenes ARqua1
Sinorhizobium meliloti ABS7/PXLDG4: Rhizobia constitutively expressing the lacZ gene

Sinorhizobium meliloti 2011
1.1 Nitrogen Fertilization

Fast growing human populations and economic forces contribute to the continuous demand for increasing agricultural yields. To meet production goals, agriculture has to use large amounts of nitrogen-based fertilizers, which has led to rampant pollution in many ecosystems (Nosengo, 2003). The equilibrium of the nitrogen cycle is threatened in many areas worldwide. Eventually the environmental and economic cost of changes may hinder prosperity of human life.

Plants growing on soil deficient for reduced nitrogen give poor yield in grains, fruits, non-reproductive parts (leaves, stem, roots), as compared to the plants growing on soils rich in reduced nitrogen. Animal or human manure, guano, salpetre are among the natural sources of reduced nitrogen applied on fields to improve plant yield (Heinonen-Tanski and van Wijk-Sijbesma, 2005; Schroder, 2005).

Natural fertilizers as the source of reduced nitrogen met the needs for growing crops until the start of the twentieth century. The extent of use of traditional fertilizers could be increased as long as they were available and affordable for farmers.

In 1909, the invention of the Haber-Bosch process for making synthetic nitrogen fertilizer revolutionized agriculture (Smil, 2002). The process made possible mass production of nitrogen fertilizer through the reduction of atmospheric nitrogen gas. Throughout the twentieth century the use of synthetic nitrogen fertilizer became
widespread. Today’s agricultural yields are largely dependent on the massive use of synthetic nitrogen fertilizer.

The large amounts of synthetic nitrogenous fertilizers applied in the fields are not entirely taken up by plants. Nitrogenous compounds such as nitrate or ammonia remaining in the soil or running off into aqueous systems can affect ecosystems in various ways (Nosengo, 2003). The microbial digestion of ammonia can lead to soil acidification. Nitrates left in the soil can bind positively charged ions. When the nitrates leach away from the soil, they take with them micronutrients such as magnesium and calcium leading to soil impoverished for positively charged nutrients. The leaching of nitrates to ground water can also cause other severe ecosystem problems (Burkart and Stoner, 2002). Excess nitrate in water system can lead to eutrophication (Rabalais, 2002). If the nitrate level is not properly controlled in water systems, drinking water is not suitable for consumption. With high level of nitrate, sicknesses such as methaemoglobinaemia may occur. Agricultural activities can cause great imbalances of the nitrogen cycle, which eventually put threat on human life and Earth ecosystem.

1.2 Symbiotic Nitrogen Fixation

Legumes are self sufficient with respect to nitrogen. Soybean, alfalfa, peanut, and beans require little nitrogen fertilizers as compared with non-legumes for reaching desired agricultural yields. Legume cash crops are beneficial in terms of the revenue they directly generate and the savings they allow. Moreover, parts of these plants, which are not harvested, are recycled in the fields. The degradation of the tissues
increases the reduced nitrogen levels in the soil, which is then used for the benefits of
the non-legume crops cultivated after legume crops. The same agricultural practice with
non-legumes is not as efficient in replenishing the soil reduced nitrogen stocks. For
centuries, the practice of including legumes in a crop rotation has been known to be
crucial for growing biomass of non-legumes.

The observed key properties of legumes partly reside in their unique ability to
benefit from the reduction of atmospheric dinitrogen into ammonia for synthesis of
reduced organic nitrogenous compounds (Brewin, 1991). Recently, it has also been
proposed that legumes may promote nitrification (Hipkin et al., 2004). Most legumes
live in association with soil bacteria harbored in nodules on their roots. The prokaryotic
partners of the legumes are responsible for the reduction of dinitrogen to ammonia.
Plant cells carry out the assimilation of ammonia into organic nitrogenous compounds.
Symbiotic nitrogen fixation (SNF) pathways hosted by legumes could be engineered in
non-legumes (Dey M Fau - Datta and Datta, ; Sanchez F Fau - Cardenas et al., ; Ladha
et al., 1997). Agricultural yields for these plants would be much less dependent on
synthetic nitrogen fertilizers than they currently are. For this revolution to happen
however, a comprehensive understanding of the establishment and maintenance of SNF
at the cellular and the molecular level is necessary.
1.3 Nodule Development

1.3.1 Nodule Formation

Root exudates chemoattract rhizobia towards the root hair cells. Nod factors from rhizobia elicit root hair cell curling which result in the entrapment of bacteria. Also cell divisions are elicited by Nod factors in the root cortex. Through an infection thread, the bacterial cells migrate toward the newly divided cells, in which they get internalized. A new organelle is created in the cytoplasm of these cells, the symbiosome which will differentiate into a nitrogen-fixing organelle. Cortical cell divisions eventually create the full nodule structure rising from the root cortex.

1.3.2 Nodule Initiation

SNF occurs when the legumes associate with the soil bacteria, rhizobia, in a symbiosis (Hirsch, 1992; Broughton et al., 2000) which requires the formation of a nodule on the roots of legumes (Figure 1.1, Panel A). Rhizobia are found in the legume rhizosphere. Signal exchanges triggers nodulation initiation between legumes and rhizobia (Figure 1.1, Panel B), which results in the formation of a root nodule (Figure 1.1, Panel C). Rhizobia are attracted towards the legume roots because of the flavonoids (Figure 1.2, Panel A) exuded by the plant roots. Some rhizobial genes are expressed when the Nod box of their promoters is concomitantly stimulated by the constitutively expressed rhizobial NodD gene product and legume flavonoids. Synthesis of Nod factors (Figure 1.2, Panel B) by rhizobial cells ensues. Secreted Nod factors
Figure 1.1 Formation of a nodule. The presence of nodules on roots of *M. truncatula* results from the interaction of *S. meliloti* and root hair cells in the rhizosphere. Panel A: *M. truncatula* and its nodulated root system. Nodules of *Medicago* emerge from a susceptible zone near the apical root meristem. As the susceptible zone moves due to root growth, nodules at different stages of organogenesis are visible on the root: from the early root hair curling and nodule primordium formation (Panel B) to a mature nodule (Panel C). Panel B: nodule initiation in *M. truncatula* root epidermis upon interaction with *S. meliloti*. Rhizobia secrete Nod factors which induce root hair cells to curl. Rhizobia get confined in an infection pocket. Inward growth of the root hair creates an infection thread: rhizobia enter the root hair cell, but are not in direct contact with its cytoplasm. Rhizobia reach newly formed primordial cells in the root cortex, in which they are internalized as symbiosomes, resembling acquired organelles. Panel C: The mature nodule contains a meristematic zone (I), an infection and early symbiosome formation zone (II), a nitrogen-fixing zone (III) and a senescence zone (IV). Starch accumulation occurs in interzone II-III (Vasse et al, 1990). In zone IV, nodule cells containing bacteroids are lysed. Because *Medicago* keeps an active apical meristem, new tissue is constantly formed. Senescence of cells from zone III is compensated by differentiation of new cells, which are invaded by rhizobia cells from the infection threads. Reduced compounds formed in the nodule tissue (-N-) are transferred from to the plant by the nodule vascular bundle (VB), which is connected to the root vascular bundle (RVB). The plant gives photosynthates (-C-) to the bacteroids as an energy source for the highly energy-demanding reduction of atmospheric nitrogen into ammonia (Adapted from Hirsch, 1992).
Figure 1.2 Flavonoid and Nod factor chemical structure. Panel A: flavonoids like apigenin (Broughton et al., 2000) can induce the transcription of the rhizobial *nod* genes, which are involved in the production of Nod factors. Panel B: generalized structure of a Nod factor (Hirsch, 1992). ‘n’ refers to the number of glucosamine residues. The lipid moiety shown is typical of Nod factor secreted by *S. meliloti*. 
induce changes in the legume roots before rhizobia and legumes come in contact. First, the root hair cells growth pattern becomes anisotropic: they bend. Nod factors also induce the dedifferentiation of cells in the pericycle and the root cortex. Cell divisions ensue: a primordium forms in the inner cortex of the root (Brewin, 2004). Before rhizobia reach the legume roots, morphological changes have started to happen in the root cortex. Rhizobia reach the deforming root hair cells, which entrap them. Within the formed root hair infection pocket a rhizobia colony starts growing. At the site of the infection pocket, the cell dissolve locally; there the plasma membrane starts to grow inward defining an infection thread in the root hair cell. The entrapped rhizobia travel down the infection thread towards the newly formed primordia in the root cortex. Bacterial are never in contact with the cytoplasm of the plant cells, but are embedded in a matrix bordered by cell wall material. Eventually rhizobia reach the vicinity of the nodule primordium cells. All infecting rhizobia are consistently targeted to a common final destination.

1.3.3 Symbiosome Formation and Bacteroid Differentiation

At the tip of the infection thread, the cell wall of a newly divided cortical plant cell dissolves: the infection thread comes in close contact with the plasmalemma (Figure 1.3). Infection droplets containing one or more rhizobia bud off from the infection thread to be engulfed by a neighboring plant cell. These new cytoplasmic structures are the symbiosomes. Internalized bacteria keep dividing within the plant cell, in conjunction with the plant membrane that surrounds them. The cytoplasm of the
Figure 1.3 Formation of the symbiosome. Rhizobia progress in the infection thread growing, which travels in the plant cell cytoplasm. Rhizobia are released in the cytoplasm during a process, which resemble endocytosis. The endocytic vesicle differentiates into a symbiosome. The rhizobia develops into a bacteroid. It is surrounded by the symbiosome membrane of plant origin and separated from it by the symbiosome space (Adapted from Whitehead and Day, 1997).
invaded cells gets packed with symbiosomes. When rhizobial divisions stop, bacteria
start to differentiate into bacteroids: the bacteria elongate and their cytoplasmic density
increases (Vasse et al., 1990; Timmers et al., 2000).

Eventually, bacteroids reach a differentiation stage at which they are able to
reduce dinitrogen into ammonia. Production of ammonia from dinitrogen is an
energetically expensive process. To reduce one mole of dinitrogen, 16 moles of ATP are
needed (Prell and Poole, 2006):

\[
\begin{align*}
\text{N}_2 + 8e^- + 8H^+ + 16\text{ATP} & \rightarrow \\
2\text{NH}_3 + H_2 + 16\text{ADP} + \text{P}
\end{align*}
\]

Bacteroids derive the necessary energy from the oxidation of dicarboxylic acids
provided by the plant. In exchange, there is a flow of reduced nitrogen reaching the
plant cells. Dinitrogen reduction marks the final stage of bacteroid differentiation.

Bacteroids eventually undergo senescence and get lysed along with the cells
which harbored them (Vasse et al., 1990; Timmers et al., 2000). For the most part,
once rhizobia are internalized in the plant root cells, they keep undergoing tremendous
changes without reaching a steady developmental state. The changes undergone by the
rhizobia from a bacteroid that fixes nitrogen to a bacteroid which senesces could be
explained by biochemical conditions in the symbiosome becoming increasingly
unfavorable to the bacteroid that fixes nitrogen (Verma and Hong, 1996; Groten et al.,
2006). It may be more beneficial for a legume to senesce parts of its bacteroid-invaded
nodule tissues than to maintain a relatively constant level of living bacteria in its tissues.
Rhizobia use nutrients from the legume to derive the large amounts of ATP necessary
for the reduction of dinitrogen into ammonia (Prell and Poole, 2006). The energy
demands driven by the nitrogen-fixing bacteria should be controlled for the plants not to become starved for the nutrients they need to insure their own growth (Nishimura et al., 2002). Interestingly, plant proteases have been hypothesized to be involved in the control of nodule senescence (Sheokand and Brewin, 2003).

1.3.4 Nodule Tissue Zones

A mature nodule of *Medicago truncatula* may contain different tissue zones (Hirsch, 1992). There is an apical meristem (zone I) where cells actively divide (Figure 1.1, Panel C). The new cells differentiate and make up the zone II. In this latter zone, infection threads harboring rhizobia grow and deliver them into the cytoplasm of plant cells. Zone II is the earliest zone in which plant cells contain symbiosomes. In zone III, the symbiosomes differentiate and are able to fix nitrogen into ammonia. Eventually, plant cells and symbiosomes from zone III senesce and lyze, defining the senescence zone IV.

The establishment and the maintenance of SNF follow a precise sequence of events which are similar between different species of legumes. The basic principles of SNF and nodulation are likely to be common to all legumes. Research efforts are now being concentrated on a model legume, *Medicago truncatula*, to dissect the molecular mechanisms of SNF. A model legume should have characteristics which help speed up SNF research.
1.3.5 Medicago truncatula: A Model Plant for SNF

The barrel medic, Medicago truncatula, from the plant family fabaceae, is being developed as a model legume for the analysis of SNF (Barker et al., 1990; Cook, 1999). It has relevant traits for genetic studies: diploidy, self-fertility, and rapid generation time allowing several life cycles per year. Routine laboratory techniques make possible the isolation of mutants defective for some aspects of nodulation. In forward genetics, mutants can be created by ethyl methane sulfonate treatment, fast neutron bombardment or other means. Consequently, the genes that caused the mutation can be mapped, cloned, and then the function of their gene product studied (Endre et al., 2002; Stracke et al., 2002; Oldroyd and Long, 2003; Ane et al., 2004; Mitra et al., 2004; Veereshlingam et al., 2004; Smit et al., 2005). Identification of nodulation genes will be facilitated by the M. truncatula genome sequencing project which is over 60% complete (Young et al., 2005). Nodulation genes can also be identified by reverse genetics through gene expression analysis (cDNA libraries, EST libraries, microarrays), and proteomics analysis (nodulin antisera, 2D gels). Last but not least, the Medicago research news are compiled and regularly updated on a publicly available website at www.medicago.org. The array of research tools available for M. truncatula makes it a very appealing model legume.

Nevertheless researchers are still yearning for additional techniques to be established in M. truncatula. For example, transformation and regeneration protocols for M. truncatula are rather lengthy and labor-intensive (Chabaud et al., 1996; Trinh et al., 1998). Non-clonal transformation of roots with A. rhizogenes is nevertheless
possible to express a transgene or to suppress gene expression by the introduction of an RNA interference cassette (Boisson-Dernier et al., 2001; Chabaud et al., 2003). Additionally, not all legumes have the same nodule organogenesis patterns and SNF-related functions as those found in _M. truncatula_. Most temperate legumes have persistent nodule meristems; such legumes are called indeterminate (Hirsch, 1992). For example, a _Medicago_ nodule can grow indefinitely because the meristem at its tip keeps functioning even as the nodule reaches the mature dinitrogen reduction stage. In a mature indeterminate nodule, the early stage of nodulation (infection threads, early symbiosome differentiation stages) are still happening: the expression pattern of early nodulin genes may overlap with the expression pattern of late nodulins. On the other hand determinate legumes are defined as legumes for which mature nodules do not have a persistent meristem, which is the case of a majority of legumes of tropical origin. As a determinate legume, _Lotus japonicus_ may be advantageous in order to study the temporal pattern of expression of nodulins.

By employing _M. truncatula_ and _L. japonicus_, as legume model plants, molecular mechanisms of SNF are being uncovered. Genes have been identified which play a role for SNF establishment and maintenance. All these genes may or may not be uniquely expressed during nodulation (Gresshoff, 2003; Silverstein et al., 2006).

1.3.6 Genetic Underpinning of Nodulation

Nodulation is dependent on nodule-specific genes and housekeeping genes which are expressed in nodules as well as in other plant organs. Expression of
housekeeping genes is necessary for the nodule to function. Expression of these genes may be enhanced during nodulation, as compared to their expression in other organs of the plant. For example, sucrose synthase is expressed in M. truncatula and soybean nodules at levels 10 to 20 times higher than in root tissues (Hohnjec et al., 1999; Zhang et al., 1999; Hohnjec et al., 2003). Other nodulation genes are specifically and only expressed during nodulation (Hirsch, 1992; Szczyglowski and Amyot, 2003). These genes may have evolved from ancestor genes which were expressed in other organs of the plants, as hinted by the increasing number of archetypal nodulins found to be expressed in non-symbiotic tissues (Hirsch, 1999). Nodule-specific genes have received a lot of attention as they may be key to unravel the mechanisms of nodulation.

Understanding nodulins’ functions may be quite crucial in the effort to import SNF to non-legume crops. Nodule-specific genes are still conveniently categorized depending on when they start being expressed once legume roots are exposed to a rhizobia inoculum. Genes expressed early during nodulation are called early nodulin (ENOD) genes. Late nodulins are usually defined by their expression being closely associated with the onset of dinitrogen reduction. The natural temporal-sounding classification of nodulins is convenient, but in some instance, may be in contradiction with the actual function of a gene and also it may leave out other symbiosis-specific genes. For example, root sites susceptible for rhizobia infection contain receptors encoded by constitutively expressed genes which are not classified as ENODs. (Etzler et al., 1999; Hirsch, 1999; Limpens et al., 2003). Also preexisting signaling-involved proteins like the mitogen-activated protein kinases (MAPK) may specifically be involved
in transducing early signal exchanges between the legume and rhizobia (Schoenbeck et al., 1999).

Early nodulins, which may be necessary not only for nodule formation but also for functionally mature nodules, could be localized in the symbiosome. I will name them symbiosins to emphasize their close association with the symbiosome.

Symbiosins are particularly important because of their location, at the very interface between the plant and the bacteroid: in the symbiosome membrane and in the symbiosome space (Figure 1.4). Known symbiosins are involved in a variety of functions (Verma and Hong, 1996; Udvardi and Day, 1997; Whitehead and Day, 1997; Catalano et al., 2004): transporters (aquaporins, e.g.: nodulin-26 (Rivers et al., 1997; Dean et al., 1999), ammonia transporters (Day et al., 2001), dicarboxylic acids transporters (Garg et al., 2004)), ATPases (Corzo et al., 1997), etc. By defining the molecular functions of symbiosins, one could possibly explain how symbiosomes are kept functional and why they eventually senesce and lyze. Nodulins and especially symbiosins are important to our understanding of nodulation in the long-term prospect of transferring SNF to non-legume crops. Much remains to be done to grow from genomic and proteomic data which keep accumulating for nodulin (Fedorova et al., 2002; Wienkoop and Saalbach, 2003; Catalano et al., 2004).
Figure 1.4 Schematic of the symbiosome. In the cytoplasm, the symbiosome contains a nitrogen-fixing bacteroid that interacts actively with its legume symbiont. Carbohydrates (e.g., malate) are channeled into the symbiosome space, which the bacteroid can use as an energy source. Ions, amino acids and water are transported across the symbiosome membrane. ATPases acidifies the symbiosome space and creates the proton gradient which drives the dicarboxylates across the membrane. Proteins of plant origin (green) may be at various location in the symbiosome: adjacent to the membrane, on the cytoplasm or symbiosome space side, transporters spanning the membrane or soluble protein of the symbiosome space (Adapted from Verma and Hong, 1996; Whitehead and Day, 1997; Catalano et al., 2004).
1.4 Background and Significance of the Early Nodulin Gene 8

1.4.1 ENOD8 cDNA Identification

ENOD8 was one the nodulins identified by screening of a cDNA library made from poly A mRNA nodules of *M. sativa* cv. Iroquois. Plant genes markers for bar nodules, and empty nodules were sought (Dickstein et al., 1991). In bar nodules, bacteria stay in infection threads: they are not released in plant cells. Such nodules are elicited on *M. sativa* roots by *hemA S. meliloti* mutants. Empty nodules are elicited by exo or ndv *S. meliloti*: they do not display normal infection thread growth. cDNA clones were selected that hybridized with nodule RNA, but not with seedling RNA (Dunn et al., 1988; Dickstein et al., 1991). One of these isolated cDNA clones, 11A, was weakly expressed in empty, bar nodules and wild-type nodules, and was not expressed in seedlings (Dickstein et al., 1991). The 11A clone was used to screen a ZAPII *M. sativa* cDNA library, from which a full-length ENOD8 cDNA was isolated (GenBank L18899). This sequence could be used for expression studies. In *M. sativa*, it was found that ENOD8 is expressed in empty, uninvaded and wild-type nodules. In *M. truncatula* mutants defective for early steps of nodule formation (infection thread formation, cell invasion), no ENOD8 mRNA expression was found (Kuppusamy et al., 2004; Veereshlingam et al., 2004). These observations suggest that ENOD8 may be involved in the nodulation stages preceding atmospheric ninitrogen reduction (Dickstein et al., 1993), which may not be the same in all legumes.
1.4.2 ENOD8 Expression

The ENOD8 mRNA is found in nodules, but not in flowers, seedpods, leaves or seedlings of *M. truncatula*. ENOD8 mRNA is detectable on northern blots at 5 days post-inoculation (dpi) with *Sinorhizobium meliloti*. The start of expression of ENOD8 falls between the onset of expression of ENOD2 (4 dpi), and leghemoglobin (6 dpi), which are markers for early nodulin gene and late nodulin expression, respectively (Dickstein et al., 2002). ENOD8 may participate in the organogenesis of the nodule. The relatively late start of expression for ENOD8 could mean that its role is not related to the onset of organogenetic events (infection thread formation, growth) but to later events such as the delivery of the rhizobia to the plant cells of the primordium, or symbiosome formation.

1.4.3 ENOD8 Sequences in *M. sativa* and *M. truncatula*

With the ENOD8 full-length cDNA (GenBank L18899) available, an extensive survey of ENOD8 sequences could be undertaken. The first ENOD8 sequences were isolated from the pasture legume alfalfa, *M. sativa*, because it was widely studied in the SNF research community. *M. sativa* was also favored as it is quite a hardy plant since it does not require high maintenance work. As the scientific community switched to the *M. truncatula* model legume it became advantageous to work with *M. truncatula* ENOD8 sequences. *M. truncatula* is also more amenable to genetic analysis than *M. sativa*.

Initial *M. sativa* sequence surveys delivered a partial ORF sequence (GenBank L31633), and a full length genomic sequence (GenBank U35073) including the promoter
(Peng and Dickstein, 1994; Kang et al., 1995). Southern analysis on *EcoR* I-digested *M. sativa* cv. Iroquois genomic DNA yields five bands hybridizing strongly with *ENOD8* and two bands that hybridize weekly with *ENOD8*. In *M. truncatula*, the same Southern experiment yields only 3 bands. The genomic complexity is likely to be less in the diploid autogamous *M. truncatula* than in the cross-fertilizing tetraploid *M. sativa*.

Consequently, a mature *M. truncatula* root nodule cDNA library was screened with the *M. sativa* *ENOD8* cDNA (GenBank L18899) and a full-length cDNA clone (GenBank AF064775) was isolated (Liu et al., 1998). The *M. sativa* *ENOD8* cDNA (GenBank L18899) was also used for screening a λ-based genomic *M. truncatula* library (Gallusi et al., 1991). Two different *M. truncatula* sequences were retrieved: a cDNA clone (GenBank AF064775), and a genomic DNA clone (GenBank AF463408) (Dickstein et al., 2002). One of the two sequences isolated, 12.7.1 (GenBank AF463408) is different from the *M. truncatula* cDNA clone (GenBank AF064775). The other sequence corresponds to that clone (GenBank AF064775).

Based on the *M. truncatula* Southern blot data, there should be more *ENOD8* genomic sequences. A bacterial artificial chromosome (BAC) library (Nam et al., 1999) was screened with the *M. truncatula* cDNA clone (GenBank AF064775) so that a complete census of *ENOD8* sequences could be achieved. A cluster of genes formed by *ENOD8.1, ENOD8.2, ENOD8.3* (GenBank AF463407) within the BAC clone 19N23 was found (Dickstein et al., 2002).

Northern blot studies for *ENOD8.1* (nt10554-12564 of GenBank AF463407), *ENOD8.2* (nt87564-89447 of GenBank AF463407) and *ENOD8.3* (nt90647-95068 of
GenBanK AC139354) demonstrated that only \textit{ENOD8.1} is expressed at significant level in nodules. Competitive RT-PCR and Southern blots of the RT-PCR products showed that the expression level of \textit{ENOD8.2} is at least four orders of magnitude lower than \textit{ENOD8.1}. No \textit{ENOD8.3} mRNA transcripts could be detected (Dickstein et al., 2002). The survey of expressed sequence tags (EST) libraries showed that only ESTs corresponding to \textit{ENOD8.1} could be detected. No ESTs were detected for \textit{ENOD8.2}, \textit{ENOD8.3} and the other three \textit{ENOD8}-like sequences, which were found by genome sequencing survey (Dickstein et al., 2002). It is therefore likely that only the \textit{ENOD8.1} sequence is expressed at high levels in nodules.

Duplication events may explain the high number of \textit{ENOD8} copies. Some copies may be mutational sinks permitting at least one \textit{ENOD8} copy to remain active. This may indicate that the \textit{ENOD8} gene function is important to nodule development and/or function.

1.4.4 Sequence Homology of the \textit{ENOD8} Predicted Protein Sequence

Through sequence homology survey of a protein, it is possible to detect amino acids consensus motifs, which could correlate with the protein biochemical function. Extracts containing the protein can then be tested against substrates characteristic for a candidate biochemical function. The sequence homology survey of the \textit{ENOD8} sequence shows a consensus motif, characteristic of the GDSL family of hydrolytic enzymes found only in plants and bacteria (Brick et al., 1995; Upton and Buckley, 1995; Akoh et al., 2004). This family contains lipases and esterases and is characterized by five
blocks of conserved sequences, including a putative serine, aspartic acid, and histidine active site. GDSL refers to the amino acid context of the putative active site serine, which is not perfectly conserved in plants.

Very interestingly, a few GDSL plant enzymes sharing high homology with ENOD8 have been identified as lipases or esterases. A lanatoside 15’-O-acetyesterase from Digitalis lanata shares 44% sequence identity with ENOD8 at the amino acid level. The acetyesterase removes an acetyl group from the substrate lanatoside A in the digitonin biosynthetic pathway (Kandzia et al., 1998). An A. thaliana lipase shares 35% identity with ENOD8 at the amino acid level. A lipase expressed in Escherichia coli, conferred lipolytic activity on the recipient strain (Brick et al., 1995). A GDSL exopolygalacturonase, a secreted glycoprotein, from Daucus carota shares 47% identity with ENOD8 at the amino acid level (Tanaka et al., 2002). EP4 and iEP4 which are 97% identical to that exopolygalacturonase are induced by a Sclerotinia sclerotiorum elicitor (van Engelen et al., 1995; Bertinetti and Ugalde, 1996). Other GDSL plant enzymes have specific expression pattern. The A. thaliana APG gene is anther-specific (Roberts et al., 1993). A Brassica napus myrosinase-associated protein is wound and methyl-jasmonate inducible (Taipalensuu et al., 1996).

As the ENOD8 protein has a putative signal peptide and N-glycosylation sites (von Heijne, 1983; Dickstein et al., 1993), we can hypothesize that ENOD8 is a secreted hydrolase which could act on an oligo- or polysaccharide acetylated extracellular molecule. The target of ENOD8 hydrolytic activity could be cell wall molecules or a small oligosaccharide (e.g.: Nod factor). Other studies have shown that esterases are
specifically expressed in nodules of legumes, including *M. sativa*, but not in their roots (Fottrell and Masterson, 1963; Fottrell, 1968). Protein extracts were run on starch electrophoresis gel in native conditions. Esterase native gel assays were then performed using general esterase substrates. Experiments were not carried out any further (Dr. Fottrell, personal communication). To test ENOD8 hypothesized esterase activity, assays were performed with similar general esterase substrates.

1.4.5 Esterase Assays on ENOD8 Proteins Purified from *M. sativa* Nodules

To perform esterase assays on *M. sativa ENOD8* proteins, a purification scheme was devised employing ammonium sulfate purification and various combination of chromatographies, such as ion exchange and affinity chromatography (Pringle and Dickstein, 2004). Also, concanavalin A chromatography was performed, as ENOD8 has several N-glycosylation sites. ENOD8 proteins from *M. sativa* were purified to homogeneity: 3-4-5 ENOD8 proteins could not be separated from each other.

Purified ENOD8 fractions were assayed by spectrophotometry. In the presence of ENOD8 the hydrolysis rate of α-naphthylacetate followed saturable kinetics. From Michaelis-Menten kinetics plots, it was calculated that the ENOD8 purified fractions have a $K_m = 50 \pm 38 \mu M$ and a $V_{max} = 6.9 \pm 1.0$ nmol/min. Activity was not observed for longer chain p-nitrophenylesters. Native gel esterase assay showed that the proteins from the ENOD8 purified fractions have hydrolytic activities toward α-naphthylacetate. Western blot analysis confirmed that these proteins were ENOD8. Biochemical assays support the hypothesis that ENOD8 is an esterase as the sequence homology survey suggested.
1.5 Objectives of this Study

A molecular biology approach was employed to elaborate on the function of the ENOD8 protein in the symbiosis between M. truncatula and S. meliloti. The genomics of ENOD8 was studied. At first the subcloning of exon 1 of ENOD8.3 (nt94820-95068 of GenBank AC139354) was attempted. Secondly the number of M. truncatula gene sequences with a high degree of homology to ENOD8.1 (nt10554-12564 of GenBank AF463407) was estimated by Southern blot analysis (Chapter 2). The expression and purification of soluble recombinant ENOD8 protein was undertaken in E. coli, P. pastoris and N. benthamiana. Recombinant ENOD8 expression enabled testing the biochemical functionality of a heterologously expressed ENOD8 protein and enabled raising an antisera against the entire ENOD8 protein (Chapter 3). Nodules from a M. truncatula line containing a sequence with the ENOD8 promoter (nt7160-10554 of GenBank AF463407) fused with the GUS gene were stained with X-gluc. Stained areas of the nodule indicate where the ENOD8 promoter directs expression (Chapter 4). An affinity-purified ENOD8 antibody isolated from an anti-ENOD8 oligopeptide antisera was used to localize the ENOD8 protein in nodule sections at the tissue and cell level. Knowing the area of the nodule in which the ENOD8 protein localizes can contribute to hone in on the biological function of ENOD8 (Chapter 5). An RNA interference strategy was used to test the effect of the absence of ENOD8 protein on nodulation in M. truncatula. Transformed M. truncatula lines were screened
for individual plants that show decreased expression in \textit{ENOD8} protein due to the presence of an \textit{ENOD8}-RNA interference cassette (Chapter 6).
CHAPTER 2
CENSUS OF THE ENOD8 GENES

2.1 Introduction

The findings of *M. truncatula* sequences with high homology for *ENOD8.1* (nt10554-12564 of GenBank AF463407) are described in this chapter. At the start of this study, the *ENOD8.1, ENOD8.2*, and *ENOD8.3* (nt2708-12707 of GenBank AF463407) genes were completely sequenced, except for the first exon of *ENOD8.3*. Discovery of the *ENOD8.1-3* gene cluster was the result of the survey of the mth1 BAC library and sequencing of 12.7 kb of BAC clone 19N23 (Yang, 1998; Nam et al., 1999; Dickstein et al., 2002). The screening of the above BAC library with the *M. sativa* *ENOD8* cDNA (GenBank L18899) showed that five BAC clones contained *ENOD8* sequences: 19N23, 31E11, 51I19, 58P7, 64N13. Subsequently, research on *ENOD8* in the Dickstein laboratory contributed to the choice of BAC clone mth1-64n13 (GenBank AC139354) for sequencing by the *M. truncatula* sequencing project (Cannon et al., 2005; Young et al., 2005).

An initial goal was to find exon 1 of *ENOD8.3* (nt94820-95068 of GenBank AC139354). From BAC clone 19N23, a 2.7 kb *Hind* III DNA fragment (nt1-2707 of GenBank AF463407), upstream of exon 2 of *ENOD8.3*, was selected. Subcloning of the *Hind* III fragment was done in the vector pBluescript II SK- (Stratagen, La Jolla, CA). With this vector it was possible to select for transformed colonies by blue/white color selection and to perform sequencing. Subsequently, a major goal of *ENOD8* research was to find all the *ENOD8* copies, which are expressed during nodulation. Experiments
were then undertaken to find unique ENOD8 sequences. Southern blot hybridizations were performed on restriction digests of BAC clones 19N23, 31E11, 51I19, 58P7, 64N13. The BAC clones hybridization results, combined with Dr. Dickstein’s genome sequencing survey database analysis and genomic Southern blots, identified six ENOD8 genes on a 70 kb DNA fragment of BAC clone mth1-64n13 (GenBank AC139354) (Dickstein et al., 2002).

Searches of databases of the national center for biotechnology information yielded additional sequences highly homologous at the amino acid level to ENOD8.1 (GenBank AAL68832). Two sequences highly homologous to ENOD8.1 and the ENOD8.1-.6 sequences were tested for their possible evolutionary relations by a phylogenetic analysis.

2.2 Subcloning of the Genomic Sequence 5’ of Exon 2 of ENOD8.3

In order to find a sequence with high homology to exon 1 of ENOD8.1 (nt83922-84170 of AC139354), gene walking was performed on BAC clone 19N23, upstream of the 5’ end of exon 2 of ENOD8.3 (nt92023 of AC139354). According to the complete genomic sequences of ENOD8.1 (nt82167-84170 of AC139354) and ENOD8.2 (nt87567-89447 of AC139354), we hypothesized that a DNA fragment over 2 kb may contain exon 1 and intron 1 of ENOD8.3 (Figure 2.1). To obtain the DNA 5’ to exon 2 of ENOD8.3, we first identified, by Southern hybridization, a 2.7 kb Hind III DNA fragment from BAC clone 19N23, which contains the first few codons of exon 2 of ENOD8.3 and
Figure 2.1 Genomic organization of the ENOD8 genes. The red arrows represent the direction of the coding sequences. The exons are numbered and represented in black. The introns are represented in blue; the number of an intron is the same as the number of the exon immediately adjacent to its left in panel B. Panel A: ENOD8.1-.6 genes are distributed over 70.2 kb of chromosome 1 of *M. truncatula*. Coding sequences have the same orientation except for ENOD8.6. Panel B: detailed exon-intron structure of ENOD8.1-.8. ENOD8.7 and ENOD8.8 are located on chromosome 7 and chromosome 8, respectively. ENOD8.1 (nt82167-84170 of GenBank AC139354), ENOD8.2 (nt87567-89447 of GenBank AC139354), ENOD8.3 (nt90647-95068 of GenBank AC139354), ENOD8.4 (nt105081-108043 of GenBank AC139354), ENOD8.5 (nt109306-113033 of GenBank AC139354), ENOD8.6 (nt43816-46532 of GenBank AC139354), ENOD8.7 (nt7814-9645 of GenBank AC150244), ENOD8.8 (nt102532-104615 of GenBank AC153125).
sequences upstream of it, presumably intron 1 and exon 1 of ENOD8.3 (Figure 2.2). Then we subcloned the 2.7 kb Hind III piece and sequenced it.

The Hind III 2.7 kb DNA sequence (nt1-2707 of GenBank AF463407) contained a sequence with no homology with the exon 1 of ENOD8.1 (nt10554-10803 of GenBank AF463407) or exon 1 of ENOD8.2 (nt5277-5529 of AF463407). Exon 1 of ENOD8.3 (nt94820-95068 of GenBank AC139354) was later found through the M. truncatula sequencing project. It turns out that the 3' end of exon 1 of ENOD8.3 was less than 100 nucleotides away from the 2.7 kb Hind III that was subcloned.

The deduced amino acid sequence of exon 1 of ENOD8.3, determined later by the Medicago genome project, shares high homology with the sequences of the first exon of each of ENOD8.1 and ENOD8.2 (Figure 2.3). ENOD8.3 expression does not occur or is undetectable in the conditions tested during nodule development (Dickstein et al., 2002). In contrast, ENOD8.1 and ENOD8.2 are expressed during nodule development although at different levels (Dickstein et al., 2002). Intron 1 of ENOD8.3 spans 2.8 kb, which is long, compared to the first intron of ENOD8.1 and ENOD8.2, which are 546 and 108 nucleotide long respectively. In the M. truncatula genome most introns are smaller than 1.0 kb. The long intron 1 of ENOD8.3 might interfere with ENOD8.3 expression.

2.3 Surveys of ENOD8 Sequences in BAC Clones

The first goal of this part of the project was to find how much of an overlap there were between the M. truncatula BAC clones inserts known to contain ENOD8
Figure 2.2 Identification of a DNA segment, which could bear the putative exon 1 of ENOD8.3. A: Multiple restriction digests of BAC clone 19N23 and Southern hybridization with the Hind III-Sac I fragment (nt2623-2702 of GenBank AF463407). Several bands hybridize strongly with the probe (red circles). The 2.7 kb Hind III band (green circle) was chosen for subcloning. Lane 2 was restricted with BamHI, lane 3 with EcoRV, lane 4 with Hind III, lane 5 with SacI, lane 7 with BamHI and Hind III, lane 8 with BamHI and SacI, lane 9 with BamHI, SacI and Hind III, lane 10 with EcoRV and Hind III, lane 11 with EcoRV and SacI, lane 12 with EcoRV, SacI and Hind III. Lanes 1, 6, 13: 1 kb DNA ladder. B: BAC 19N23 segment with exon 2 to exon 5 of ENOD8.3. Restriction sites shown correspond to restriction digests for the Southern hybridization (panel A). The Hind III-Sac I fragment (red rectangle) overlaps a known sequence of ENOD8.3 and a contiguous sequence upstream of it, which could bear exon 1 and intron 1 of ENOD8.3.
The potential signal sequence cleavage site (ENOD8*) and N-glycosylation sites (\(\ast\)) are shown in bold underlined upper-case letter (The amino acid shaded in yellow is glycine). Amino acids identical in the ENOD8-1-8 are framed in a black box. The catalytic triad (S, D, H) characteristic of the GDSL enzyme family is conserved for all ENOD8 copies; each residue is bold underlined upper-case letter and surmounted by \(\ast\). The potential signal sequence cleavage site (\(\ast\)) and N-glycosylation sites (\(\ast\)) are shown in bold underlined lower-case. Dashes indicate a gap in the sequence that have been introduced to maximize alignment. Vertically aligned arrowheads indicate the exon/exon boundaries. The sequence corresponding to the ENOD8 peptide sequence is underlined with a square bracket.
sequences: 19N23, 31E11, 51I19, 58P7, 64N13 (Yang, 1998; Nam et al., 1999; Dickstein et al., 2002). Because of library redundancy, several BAC clones would likely have very similar inserts, corresponding to the same genomic sequence. The second goal was to identify unique ENOD8 sequences. This BAC survey was undertaken, because, in addition to the ENOD8.1-.3 gene cluster (GenBank AF463407), Southern blot data suggested that other ENOD8 genes were likely to be found in the M. truncatula genome (Yang, 1998). BAC clones were restriction digested with Hind III and EcoR 1 (single and double digest) for subsequent analysis by Southern blot hybridization. BAC blots were sequentially probed with each full BAC (vector and insert), then probed with the M. truncatula cDNA (GenBank AF064775). By using a whole BAC DNA to probe a BAC blot, all the sequences (insert and vector) from the probe would hybridize with blot bands containing similar BAC sequences. Therefore on the BAC blot, lanes with DNA from different BAC clones might show identical hybridization bands (single bands or group of consecutive bands). These hybridization bands should form a continuous sequence on the corresponding BAC inserts. Analysis of the hybridization patterns shows that BAC clones can be categorized in two groups: (1) 19N23, 51I19, 64N13 and (2) 31E11, 58P7 each overlaps and forms a contig (Figure 2.4). Hybridization of the same series of BAC digests with the ENOD8 M. truncatula full-length cDNA (AF064775) led to the identification of five distinct DNA segments with homology for ENOD8 (Figure 2.5). All these ENOD8 sequences are included in BAC clones 19N23, 51I19 and 64N13 inserts. BAC clones 31E11 and 58P7 contains an ENOD8 sequence, also found in the 19N23, 51I19 and 64N13 inserts. To verify that the
Figure 2.4 BAC DNA Southern blot analysis with BAC probes. *Hind* III digests of BACs 25K17 (25), 31E11 (31), 58P7 (58), 74M24 (74), 19N23 (19), 51I19 (51) and 64N13 (64) and the corresponding Southern blots. Lanes were not originally adjacent to each other on gel. Lanes were digitally placed adjacent to each other. The colored vertical intervals indicate groups of bands within a lane. Groups of bands which appear to be common between BACs on a blot, should form a contiguous DNA sequence (green, yellow, orange intervals). Bands which do not hybridize with the BAC probe are indicated by red-dashed intervals. Band which hybridize with the BAC probe but do not appear in its corresponding digest lane are indicated with white-dashed intervals. The deduced BAC overlap is below the gel-blot pair. Colored sequences correspond to colored intervals for a given gel-blot pair and but are not scaled to fit the added base pair length from the corresponding bands within a colored interval. The assumption is made that each band from each BAC digest correspond to a unique sequence, i.e.: it is not repeated elsewhere in the genome of *M. truncatula*. A: hybridization with BAC 19N23. B: hybridization with 64N13. C: Analysis of both gel-blot pairs shows that inserts from BAC clones 31E11, 58P7, 19N23, 51I19, 64N13 form a contiguous sequence. BACs 25K17 and 74M24 were known to contain no *ENOD8* sequences and were used as negative controls. BACs 19N23, 51I19, and 64N13 shares large amount of DNA. BACs 31E11 and 58P7 bear common sequences, which overlap with the green contig.
Figure 2.5 BAC DNA Southern blot analysis with an ENOD8 cDNA probe (GenBank AF064775). BAC 25K17 (25), 31E11 (31), 58P7 (58), 74M24 (74), 19N23 (19), 51I19 (51) and 64N13 (64) were digested with Hind III. Panel A: Southern blot, Panel B: gel with superimposed transparent Southern blot, only the Southern bands hybridizing strongly are visible. Black dots indicate bands which hybridize with the probe. White dots mark bands which hybridize because they share homology with the probe but do not bear ENOD8 sequences (1636 bp band of ladder and 7507 bp band, the pBeloBAC11 vector). All ENOD8 bands can be found in BACs 19N23 (19), 51I19 (51) and 64N13 (64): green area. BACs 31E11 and 58P7 have the same ENOD8 bands (yellow), which is also found in BAC 19N23, 51I19 and 64N13.
identified ENOD8 bands from the BAC clones did correspond to actual sequences in the *M. truncatula* genome, Southern blot analysis of Hind III digest of 64N13 and *M. truncatula* genomic DNA with the ENOD8 *M. truncatula* full-length cDNA (AF064775) was performed. With high stringency hybridization conditions the five ENOD8 bands from the BAC 64N13 were found in *M. truncatula* genomic DNA (Figure 2.6). These bands must correspond to DNA fragments containing large portions of ENOD8 genes. Other fragments, which might be the smaller bits of these ENOD8 genes were revealed by hybridization performed at low stringencies.

A Hind III electronic digest of the sequence reported for mth1-64n13 (GenBank AC139354) was compared to an actual Hind III digest of mth1-64N13. The digests have an identical band pattern (Figure 2.7), supporting the results for ENOD8 sequences in BAC mth1-64N13. Digestion results and the genome sequence survey of BACs mth1-51I19 and mth1-64N13 carried out by Dr. Dickstein indicated that as many as six ENOD8 genes with the full complement of exons and introns could be found on one BAC clone, 64N13 (Dickstein et al., 2002).

2.4 Phylogeny of the ENOD8 Copies in the *M. truncatula* Genome

The ENOD8.1-6 genes are located within 100 kb from each other on chromosome 1 (Choi et al., 2004). A tblastx search for homologs of the ENOD8.1 protein sequence (GenBank AAL68832) on the *M. truncatula* nr database of the national center for biotechnology information (NCBI, www.ncbi.nlm.nih.gov/) returned two other gene sequences (Figures 2.1, 2.3). The international *Medicago* genome annotation
Figure 2.6 BAC and *M. truncatula* genomic Southern blot analysis. BAC 64N13 (BAC) and *M. truncatula* genomic DNA were digested with *Hind* III. Both digests were probed at high (‘high’) and low (‘low’) stringencies conditions with the *ENOD8* cDNA (GenBank AF064775). *ENOD8* bands previously identified in 64N13 appear also in the genomic digest (white dots). At high stringencies, additional bands appear (white squares).
Figure 2.7 BAC mth1-64n13 *Hind* III electronic digest and *Hind* III digest of BAC 64N13. Matching bands of both digests are indicating by alternating green and red dots. Two red rectangles mark areas of the gel where bands did not resolve individually. ‘v’ indicates the vector band on the gel. The electronic digest does not contain vector sequence. A 1 kb ladder is added at the left of each digest. The pattern of dots and rectangles is identical between both digest (except for the vector bands). Sequence of BAC 64n13 is likely to be identical to the sequence of mth1-64n13.
group (IMGAG) categorized the conceptual translations of both genes (GenBank ABD32376 and GenBank ABE87642) as ‘GDSL lipolytic enzymes’. Both ENOD8 homolog genes, ENOD8.7 and ENOD8.8, were on BAC inserts (GenBank AC150244, GenBank AC153125) with genetic markers anchoring them to chromosome 7 and chromosome 8, respectively. Additionally, 5.3 cM away from the clone BAC clone (GenBank AC139354) containing ENOD8.1-.6, another cluster of four GDSL genes (ABE88946, nt44322-45789; ABE88957, nt90978-94540; ABE88958, nt95168-95529; ABE88960, nt109323-113453) was found to be on a single BAC clone (GenBank AC146790). Sequence similarity search between ENOD8.1 (GenBank AAL68832) and three of the four predicted proteins from the GDSL genes cluster with the ‘BLAST 2 sequences’ (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) on-line NCBI tool returns 30% similarity or less. No significant similarity between one of them, the smallest of these four GDSL predicted proteins, (GenBank ABE88958) and ENOD8.1 was found.

A phylogenetic tree was made for the amino acid sequences with the highest homology for ENOD8.1 (GenBank AAL68832): ENOD8.1-.8. The tree would characterize possible evolutionary relations between these homologs (Figure 2.8). Non-ENOD8 proteins (M. truncatula GDSL-like protein, GenBank ABE94452; Rhodopirellula baltica GDSL lipase, GenBank CAD75621; Chlorobium limicola isomerase, GenBank ZP_00512185) were included in the phylogram as controls. These non-ENOD8 sequences are not expected to cluster with the other ENOD8 sequences in the phylogram. The results are presented in Figure 2.8. The branches of the phylogram are drawn so that their lengths are proportional to the evolutionary distance along that
Figure 2.8 Phylogenetic tree of the ENOD8.1-.8 predicted proteins. The tree was built with ClustalW (www.ebi.ac.uk/clustalw/). ENOD8.1 (GenBank AAL68832), ENOD8.2 (GenBank AAL68831), ENOD8.3 (Exon 1: conceptual translation of nt94820-95068 of GenBank AC139354 and Exon 2-5 GenBank AAL68831), ENOD8.4-.6 (conceptual translations: nt107765-108043 of GenBank AC139354, nt112773-113033 of GenBank AC139354, nt43816-44082 of GenBank AC139354), ENOD8.7 (GenBank ABD32376), ENOD8.8 (GenBank ABE87642), M. truncatula GDSL-like protein - ‘GDSL like’ (GenBank ABE94452), GDSL-1 (GenBank ABE88946), GDSL-2 (GenBank ABE88957), GDSL-3 (GenBank ABE88958), GDSL-4 (GenBank ABE88960), Rhodopirellula baltica GDSL lipase - ‘GDSL’ (GenBankCAD75621), Chlorobium limicola isomerase - ‘Isomerase’ (GenBank ZP_00512185). Non-ENOD8 sequences were used as a control to test the strength of the similarity between all the ENOD8 sequences. The branches are drawn so that their lengths are proportional to the evolutionary distance along that branch. ENOD8.1-.4 make a tight cluster, whereas the other ENOD8 show more sequence divergence. Nevertheless, other non-ENOD8 proteins cluster away from all know ENOD8 proteins sequences, indicating that these are likely to be closely related, by descending from a common ancestor.
branch. The ENOD8.1-.6 predicted proteins are distributed in three clades: (ENOD8.1, ENOD8.2), (ENOD8.3, ENOD8.4), and (ENOD8.5, ENOD8.6). ENOD8.7 and ENOD8.8 are not part of the (ENOD8.1-.6) clade. ENOD8.1 forms a clade with ENOD8.2 showing that these two genes have a common ancestor. These genes are both expressed. The clade formed by ENOD8.3 and ENOD8.4, whose predicted proteins have not been shown to be expressed during nodule development, are very close to the clade formed by ENOD8.1 and ENOD8.2. The ancestor of ENOD8.3 and ENOD8.4 might have had sequence change(s) impairing their expression during nodule development.

In the phylogenetic tree, the (ENOD8.5, ENOD8.6) clade has an intermediate position between the (ENOD8.1, ENOD8.2) and (ENOD8.3, ENOD8.4) clades and the ENOD8.7 and ENOD8.8 genes. These latter ENOD8 homologs have sustained the most extensive genetic change, as compared to the ENOD8.3 sequence, which is closest to the root of the tree. ENOD8.7 and ENOD8.8 might have descended from the same ancestor gene as ENOD8.1-.6. Nevertheless, the similarity that ENOD8.7 and ENOD8.8 share with other ENOD8 proteins might not just be due to generic GDSL amino acid motif(s), but to specific ENOD8 sequence motifs. Indeed, the non-ENOD8 GDSL sequences (GenBank ABE94452, GenBank CAD75621, GenBank ABE88946, ABE88957, ABE88958, ABE88960) and the isomerase sequence (GenBank ZP_00512185) form a clade, which does not include the ENOD8.1-.8 predicted protein sequences. These non-ENOD8 proteins have the least divergence with the ENOD8.7 and ENOD8.8 proteins. The corresponding ENOD8.7 and ENOD8.8 genes are not on chromosome 1 like the ENOD8.1-.6, but on chromosomes 7 and 8. The common ancestor to ENOD8.7 and
*ENOD8.8* may have originated from the common ancestors of *ENOD8.5* and *ENOD8.6* through non-homologous recombination events on chromosome 7 and chromosome 8.
CHAPTER 3
EXPRESSION AND ESTERASE ASSAY OF RECOMBINANT ENOD8 PROTEINS

3.1 Introduction

Sufficient amounts of ENOD8 protein were needed to test its activity toward acetylated extracellular carbohydrate substrates and to produce a new antisera.

The investigation of the Pichia pastoris, E. coli, and Nicotiana benthamiana recombinant systems for the expression of the ENOD8 protein is described in this chapter. An E. coli recombinant system had sufficiently high expression levels of soluble ENOD8 protein for testing its esterase activity and raising a new anti-ENOD8 antisera. ENOD8 was also successfully expressed in N. benthamiana.

3.2 Pichia pastoris Expression System

3.2.1 Expression System Characteristics

The yeast Pichia pastoris combines the advantages of a single cell expression system with the expression features of a eukaryotic organism: protein folding, protein processing, and post-translational modifications. In particular, a recombinant ENOD8 protein expressed by Pichia could get glycosylated. This is important, as the native ENOD8 protein from M. sativa is most likely glycosylated, which could play a role in its biochemical activity (Pringle and Dickstein, 2004). The Pichia pastoris alcohol oxidase (AOX1) promoter, which is tightly under the induction control of methanol, drives the expression of the foreign gene of interest. Once the AOX1 promoter is induced by methanol, robust transcription of the foreign gene ensues. According to Invitrogen
approximately 5% of the polyA⁺ RNA results from the transcription of the AOX1 gene. Sufficient Pichia biomass is obtained by growth on glycerol-supplemented media; during this phase, no AOX1 expression is expected. After enough biomass is obtained, the glycerol-containing media is replaced by the methanol-containing media to induce recombinant expression for the protein of interest. In the Pichia expression system kit from Invitrogen, the gene encoding resistance to the antibiotic zeocin is part of the recombinant DNA sequence integrated in Pichia transformants. Screening for zeocin resistance should result in selecting Pichia transformants which do have the expression cassette for the gene of interest. Selection of transformants by antibiotic is preferable to yeast auxotrophic markers which often results in selecting false positive transformants, due to auxotrophic mutant revertants. The recombinant protein can be tailored for secretion in the growth media. This is helpful in terms of protein purification, as Pichia does not excrete many proteins in its growing media. To achieve secretion, the foreign cDNA of interest is fused with the α factor prepro peptide secretion signal from Saccharomyces cerevisiae.

3.2.2 Cloning Strategy

The M. sativa cDNA (GenBank L18899) was chosen as the sequence for engineering a Pichia recombinant expression system for the ENOD8 protein. To achieve secretion of ENOD8 in the growth media, the α factor prepro peptide secretion signal sequence from Saccharomyces cerevisiae replaced the predicted ENOD8 signal
sequence (nt16 to 99 of GenBank L18899). The *M. sativa ENOD8* cDNA sequence (GenBank L18899) was therefore inserted in the pPIC\textalpha{}C vector (Invitrogen, Carlsbad, CA), immediately adjacent to the 3’ end of the \textalpha{} factor prepro peptide secretion signal sequence. The resulting recombinant secreted *ENOD8* protein would correspond to the translated product of nt100 to 1158 of the *ENOD8* cDNA (GenBank L18899). First, *Xho I* and *Not I* were engineered by PCR to the 5’ end and 3’ end of the *ENOD8* sequence (nt100 to nt1158 of GenBank L18899), respectively. Both restriction sites were immediately at the 5’ and 3’ end of the PCR product. In such cases, the efficiency of digestion by restriction enzymes is greatly reduced. The PCR product was cloned in a pUC18 vector first, instead of cloning it directly in the pPIC\textalpha{}C vector (Figure 3.1). The *Xho I-Not I ENOD8* fragment was ligated in the *Sma I* site of pUC18 and chemically transformed in competent *E. coli* (XL1Blue, Stratagen). A putatively transformed colony was selected through a Southern colony blot hybridization screen. The sequence of the colony was verified by DNA sequencing, resulting in the choice of a strain carrying pUC18 with the *ENOD8* sequence bordered by the *Xho I* and *Not I* restriction sites. The *Xho I-Not I* fragment digested from the pUC18 construct was then ligated into the pPIC\textalpha{}C vector and chemically transformed into competent *E. coli* (XL1Blue, Stratagen). The *ENOD8* insert sequence of the pPIC\textalpha{}C construct was verified by sequencing from a colony screened by blue/white colony selection. The pPIC\textalpha{}C construct was then chemically transformed in various *Pichia pastoris* strains, which were made chemically competent by PEG treatment with the *Pichia EasyComp™ Kit*
Figure 3.1 Cloning strategy for the Pichia pastoris ENOD8 recombinant system. Panel A: the ENOD8 cDNA sequence was flanked by PCR with a Xho I site at its 5’ end and a Not I site at its 3’ end. The blunt-ended PCR product was ligated with the Sma I-linearized pUC18 vector. Panel B: the Xho I-Not I fragment, including the ENOD8 sequence, digested from the pUC18 construct was ligated into the pICZαC vector. Panel C: the pPICZαC construct was transformed into various Pichia pastoris strains; it is expected to be integrated in the genome of Pichia pastoris by homologous recombination between the AOX1 promoter sequences common to the pPICZαC construct and the yeast genome. Panel D: ENOD8 expression cassette after its integration in the genome of Pichia pastoris.
(Invitrogen, Carlsbad, CA). The screening for putative recombinant *Pichia* strains was done by growing the *Pichia* transformants on zeocin-supplemented media.

3.2.3 Analysis of *Pichia* Transformants

Recombinant *ENOD8* expression was tested in two *Pichia* strains: wild-type (X-33) and a mutant strain (GS115) which is the most commonly used *Pichia* strain for recombinant (Higgins and Cregg, 1998). Additionally, the *P. pastoris* recombinantly expressing secreted albumin (Invitrogen, Carlsbad, CA) was in the GS115 strain, which was used as a positive control for recombinant expression. The GS115 strain has a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. GS115 will grow on complex medium supplemented with histidine (e.g.: minimal methanol media with histidine). Screening for *ENOD8* expression was done by SDS-PAGE and western blot analyses. *ENOD8* proteins could not be detected in the cellular fraction or in the growth media of the methanol-induced *Pichia* transformants. Secreted albumin in the growth media was detected on Coomassie-stained SDS-PAGE for a *Pichia* control strain, engineered to express albumin (Invitrogen). This expression result from this *Pichia* control strain indicated that methanol-induction of the AOX1 promoter was possible in these growth conditions. The zeocin-resistant *Pichia* strains were subsequently tested by PCR to verify that the *ENOD8* cassette was present in the genome (Figure 3.2). *Pichia* strains returning positive results from the PCR test were tested for expression of *ENOD8* protein by SDS-PAGE (Figure 3.3). No *ENOD8* proteins
Figure 3.2 PCR detection of the *ENOD8* expression cassette in transformed *Pichia* clones. ‘GS115’: *Pichia* strain used for making the recombinant strains. ‘GA1’, ‘GA2’: putative recombinant strains transformed with expression cassette devoid of *ENOD8* sequence. ‘G#5.1-.6’: putative recombinant strains transformed with *ENOD8* expression cassette. ‘#5’: expression plasmid carrying the *ENOD8* cassette. ‘L’: 1 kb ladder; ‘C’: PCR control lane: no template. The primers can amplify two target sequences: the native *AOX1* *Pichia* gene and the *ENOD8* expression cassette. Only the 2.2 kb band (‘*AOX1*’) corresponding to the native *AOX1* gene is detected in the GS115 strain. The GA2 control strain has the native *AOX1* gene and a 590 bp product (‘CC’). The smaller band corresponds to the expression cassette without *ENOD8* sequence; PCR did not work for strain GA1. In addition to the native *AOX1* band, G#5.1-.6 strains have an additional 1.6 kb band (‘EC’) corresponding to the *ENOD8* expression cassette, which co-migrates with the amplification product of the *ENOD8* expression plasmid used to transform *Pichia*. Any of the G#5.1-.6 *Pichia* strain should be carrying the *ENOD8* expression cassette and therefore could express the *ENOD8* protein.
Figure 3.3 SDS-PAGE analysis of *Pichia* strains carrying the *ENOD8* expression cassette. 'GS115': *Pichia* strain used for making the recombinant strains. 'GA2': recombinant strain with expression cassette devoid of *ENOD8* sequence. 'G#5.4': recombinant strain with *ENOD8* expression cassette. 'Galb': *Pichia* strain expressing albumin as a secreted protein. Analysis was carried on unconcentrate supernatant from the growth media ('S') and supernatant from the growth media, subsequently concentrated by ultrafiltration ('Sc'). Ladder ('L'): number refers to band size in kDa. Only the albumin ('Alb') is secreted in the growth media. Albumin is detectable in unconcentrated growth media supernatant. No *ENOD8* protein is detectable in the growth media supernatant, whether it was concentrated or not.
could be detected in total protein extracts from the selected *Pichia* recombinant strains carrying the *ENOD8* expression cassette.

The heat shock PEG transformation method on *Pichia pastoris* strains produces predominantly transformants with only a single copy of the recombinant expression cassette likely to be integrated in the yeast genome. Multiple copies of the *ENOD8* expression cassette would have been more likely to be integrated in the genome of *Pichia* by electroporation, which might have enhanced the expression levels of *ENOD8*.

3.3 *E. coli* Maltose Binding Protein Expression System

3.3.1 Expression System Characteristics

Previous *E. coli* expression systems had been successful in synthesizing the *ENOD8* protein, even though biochemical activity of *ENOD8* could not be tested (Dickstein et al., 2002). The *ENOD8* protein was expressed at low levels and was insoluble. The maltose binding protein (MBP) *E. coli* expression system kit from New England Biolabs was reported to be successful in expression and purification of functional proteins, which could not be isolated in a soluble form from other expression systems (di Guan et al., 1988; Kapust and Waugh, 1999). With the MBP expression system the MBP-*ENOD8* protein fusion could be directed to the periplasm of the bacteria where it could be easier to purify from the rest of the *E. coli* proteins. Additionally, the periplasm is a less reducing environment than the cytoplasm. Transfer of the MBP-*ENOD8* protein in the periplasm could result in the formation of disulfide bonds, which are predicted to exist in the *ENOD8* protein (Dickstein et al., 1993). After
purification of the MBP-ENOD8 fusion protein by affinity chromatography with an 
amyllose resin, factor Xa would be used to cleave off the MBP domain, in order to work 
with the protein of interest itself only.

3.3.2 Cloning Strategy

The *M. sativa* putative secretion signal sequence (nt16 to 99 of GenBank 
L18899) could interfere with transport of an MBP-ENOD8 fusion protein to the periplam 
of the recombinant bacteria. Accordingly, the *M. sativa* ENOD8 cDNA sequence (nt100 
to nt1158 of GenBank L18899) was engineered by PCR to add a *Sma* I site to its 5’ end 
and a *Sal* I site to its 3’ end. For the corresponding primers, 4 nucleotides were added 
directly adjacent to the 5’ site of the *Sma* I and *Sal* I restriction site. The restriction sites 
would otherwise be at the very end of the PCR product, which could result in very low 
efficiency of digestion. The digested *Sma* I-*Sal* I PCR product was then ligated with the 
vector pMAL-p2X, which had been digested with *Xmn* I and *Sal* I. The corresponding 
construct would be in frame the *malE* sequence coding for the MBP protein and the 
sequence coding for the ENOD8 protein (Figure 3.4). The MBP domain and the ENOD8 
domain of the fusion protein is connected by the peptide sequence IEGR, which is 
cleaved by factor Xa resulting in the separation of the MBP tag and the ENOD8 protein. 
A blue/white colony screen was performed as an initial screen to select putative 
transformants. Plasmid restriction digests analysis indicated which transformants might 
carry the correct expression vector.
Figure 3.4 MBP-ENOD8 expression construct. Sma I and Sal I restriction sites were added by PCR to the ENOD8 cDNA at its 5’ end and 3’ end, respectively. The digested PCR product was ligated with the pMal-p2X vector, digested with Xmn I and Sal I. The ENOD8 sequence is therefore in frame with the malE sequence coding for the maltose binding protein (MBP). Expression of the MBP-ENOD8 sequence is under the control of the pTac promoter, which is strongly repressed by the lacI gene product, in the absence of IPTG. The MPB-ENOD8 fusion protein should get expressed when the recombinant E. coli strain is exposed to IPTG.
3.3.3 Western Colony Blot Screening

The expression of the MBP-ENOD8 fusion protein is under the control of the pTac promoter. Without IPTG, the pTac promoter is strongly repressed by the Lac repressor, encoded by lacI, which is in the vector construct. A western colony blot was performed on induced transformant E. coli colonies, with the anti-ENOD8 oligopeptide antisera. Most of the induced colonies showed a staining pattern supporting the hypothesis that they indeed made ENOD8 protein when induced with IPTG (Figure 3.5). One of these strains was sequenced for its expression cassette and used for further expression studies.

3.3.4 Lipolytic Plate Assay

To detect hydrolytic activity in the E.coli strain that expressed MBP-ENOD8 fusion, lipolytic plate assays were undertaken. Tween 20, or Tween 80 was added to the bacterial growth media (Sierra, 1957). Tweens are water-soluble high fatty acid esters of polyoxyalkylene derivative of sorbitans (Figure 3.6). Tween molecules differ by the length of their fatty acid chain. The primary fatty acid chains are oleic acid and lauric acid (with remainder primarily of myristic, palmitic and stearic acid) for Tween 80 and Tween 20, respectively. Growth of the Pseudomonas aeruginosa lipolytic positive control strain on Tween 20 and Tween 80–supplemented plates resulted in the hydrolysis of the Tween substrates visualized by halos around bacterial colonies due to the formation calcium salt crystals. The ENOD8 strain colonies did not form halos and no crystals could be seen upon examination of the plates with a stereomicroscope. The
Figure 3.5 Colony immunoblot analysis to detect *E. coli* clones expressing *ENOD8*. Detection was performed by alkaline phosphatase staining. Two sets of membranes lying on media without IPTG (‘uninduced’), or with IPTG (‘induced’) had been streaked with putative *E. coli* transformants. On both blots, alkaline phosphatase purple staining is visible. Nevertheless, most colonies on the ‘induced blot’ are surrounded by a darker purple outline, not detected for the colonies on the ‘uninduced blot’. As an example, colony #6 signal (‘#6’) is magnified, showing a dark purple outline only on the ‘induced blot’. This staining pattern difference between the two blots suggest that most of the *E. coli* clones probably synthetized recombinant *ENOD8* protein.

Figure 3.6 Chemical structure of polyoxyalkylene derivative of sorbitans (Tween). All Tween molecules have the sum of $w, x, y, z = 20$. They differ with respect to their fatty acid chain. Tween 80’s fatty acid chain is primarily oleic acid. Tween 20’s fatty acid chain is predominantly lauric acid (with remainder primarily of myristic, palmitic and stearic acid).
structure of Tween molecule may be incompatible with ENOD8 esterase activity, despite the fact that they are esters. ENOD8 purified from nodule was shown to have activity on \( p \)-nitrophenyl esters whose fatty acid chain is no more than 4 carbon long and on \( \alpha \)-naphthylacetate (Pringle and Dickstein, 2004). Induced MBP-ENOD8 recombinant \( E. coli \) colonies were tested for their activity on \( \alpha \)-naphthylacetate, extrapolating the staining protocol from the principles of staining a native gel for activity on \( \alpha \)-naphthylacetate (Pringle and Dickstein, 2004). Colonies grown on Whatman filter paper were transferred on media containing IPTG. After induction, the filter paper was transferred in a sodium phosphate buffer solution containing \( \alpha \)-naphthylacetate. To visualize the products of a possible esterase activity, the filter paper was transferred in a solution containing fast blue B. It was expected to observe a violet color only around the bacterial MBP-ENOD8 colonies. There was no difference between the MBP-ENOD8 strain and the negative control strain carrying the expression control without the ENOD8 cDNA sequence.

3.3.5 Expression and Purification of the MBP-ENOD8 Protein

A pilot expression experiment was performed to demonstrate by western blotting that the MPB-ENOD8 fusion protein was expressed in the \( E. coli \) strain whose expression cassette was sequenced (Figure 3.7). The anti-ENOD8 oligopeptide antisera recognized a band on the membrane which corresponded to the size of the MBP-ENOD8 fusion protein, and appears only in the soluble protein fraction of induced strain carrying the ENOD8 expression construct. On Coomassie-stained SDS-PAGE, total proteins from the strain induced with IPTG showed a band whose size corresponded to
Figure 3.7 Western blot analysis of induced and uninduced MBP-ENOD8 E. coli strains. The ENOD8 antisera was used on total protein extracts from a recombinant E. coli clone (‘#6’) hypothesized to synthesize the MBP-ENOD8 fusion protein, according to the result of the colony blot (Figure 3.3). An E. coli clone transformed with the expression construct not including the ENOD8 sequence was used as an induction control; no ENOD8 protein should be synthesized in the presence or in the absence of IPTG (inducer) for strain p2X. In absence of IPTG (‘uninduced’) no ENOD8 protein is detected for strain #6. In presence of IPTG (‘induced’) the ENOD8 protein is detected as a purple band (‘ENOD8’) for strain #6. No purple band is detect for p2X, confirming that no native E. coli protein is responsible for the purple band observed for #6. The #6 strain synthesizes ENOD8 protein only when IPTG is present.
the size of the MBP-ENOD8 fusion protein. The amount of soluble fusion protein was small as compared to the amount of insoluble protein. Levels of MBP-ENOD8 in the periplasm were below detection levels, even when the protein periplasmic fraction was concentrated by ultrafiltration. The MPB-ENOD8 fusion protein was purified from the protein soluble fraction by affinity chromatography with amylose resin (Figure 3.8). Bacterial biomass from induced culture of E. coli MBP-ENOD8 recombinant strain was recovered by centrifugation. After resuspension, the bacteria were lysed by sonication, or in the case of very large amounts of bacterial material with a French press. The lysed solution was centrifuged to recover the supernatant. The extract was then mixed with amylose resin. Because of its maltose binding protein domain, the MBP-ENOD8 protein should be the only recombinant protein binding to the amylose beads. After washing the column with buffer to remove contaminants, maltose was used to elute the MBP-ENOD8 protein from the amylose beads. The beads have higher affinity for maltose than for MBP. Purified fractions of MBP-ENOD8 protein could be used for subsequent assays.

3.3.6 Spectrophotometric Esterase Assays

ENOD8 proteins purified from nodules had been shown to hydrolyze general esterase substrates: α-naphthylacetate and p-nitrophenylacetate (Pringle and Dickstein, 2004). We first wanted to test whether or not the recombinant MBP-ENOD8 fusion protein also had esterase activity on the same substrates. Factor Xa was incubated with the MBP-ENOD8 fusion for cleaving off the MBP domain away from the ENOD8 domain.
Figure 3.8 SDS-PAGE analysis of proteins fractions from pellets of induced strain #6. ENOD8 recombinant E. coli strain #6 had been induced with IPTG. Ladder ('L'): number refers to band size in kDa. The total protein fraction in presence of IPTG shows an additional band ('ind.' lane, black rectangle), not visible in the absence of IPTG ('un-ind'). The soluble fraction ('sol.') does not show high levels of expression of the MBP-ENOD8 protein; most of the expressed MBP-ENOD8 is found in the insoluble fraction ('insol.' lane, black rectangle). Sufficient MBP-ENOD8 is however present in the soluble fraction. MBP-ENOD8 was purified in large amounts by passing the induced soluble protein fraction through an amylose affinity chromatography column.
Incubation yielded only partial digests: mixture of uncleaved MBP-ENOD8 fusion (~90kDa), MBP (~50kDa) and ENOD8 (~40kDa) proteins. SDS-PAGE and western blot analysis showed that there was only a small amount of free ENOD8 protein resulting from digestion with factor Xa. A large of amount of MBP-ENOD8 was left uncleaved. Spectrophotometric esterase assays were performed on mixtures of free ENOD8 and MBP-ENOD8: no significant activity could be detected, as compared to a porcine liver esterase positive control.

3.3.7 SDS-PAGE Esterase Assay

Heterologous expression in E. coli often lead to misfolding of the expressed recombinant protein (Baneyx and Mujacic, 2004). The pTac promoter used to express MBP-ENOD8 may be responsible for the large amounts of insoluble MBP-ENOD8 synthesized. The rate of synthesis of MBP-ENOD8 may result in a titration of the folding factors necessary for ENOD8 to reach its conformation compatible with esterase activity. We wanted to test whether or not the MBP-ENOD8 fusion could be re-folded to a native conformation, which could bring back esterase activity. Samples were run on SDS-PAGE gel, and subsequently denatured by incubation of the gel in 6 M urea. Renaturation of the samples was done by incubating the gel for 15 min in phosphate buffer solutions with decreasing by half the urea concentration, from 3 M to 0.05 M. A porcine esterase was included as a positive control and showed esterase activity as visualized with p-nitrophenylacetate substrate followed by fast blue B staining (Figure
Figure 3.9 SDS-PAGE esterase assay for the MBP-\textit{ENOD8} protein. The fusion protein was isolated from recombinant \textit{E. coli}. Panel A: SDS-PAGE gel after staining with Coo-massie. Panel B: SDS-PAGE gel stained for esterase activity. Three sets of proteins were tested: a porcine liver esterase was used for positive control ('esterase control'), the \textit{ENOD8}-MBP fusion protein incubated with factor Xa ('factor Xa') or not ('no factor Xa'). Each set was treated as follows: the first three lanes of a set were treated with sample buffer containing DTT and left on ice (first lane), heated at 65°C for 2 min (second lane), boiled for 2 min (third lane); protein in the fourth lane were mixed with sample buffer without reducing agent and left on ice. Bovine serum albumin ('BSA') was used as a negative control. Ladder ('L'): numbers refer to band sizes in kDa. Both gels were loaded with identical samples. Incubating the SDS-PAGE gel with an esterase substrate should results in the formation of bands with a dark brown color, if the corresponding proteins have esterase activity. In the presence of factor Xa, the \textit{ENOD8}-MBP fusion protein is partially cleaved into its two subunits: MBP and \textit{ENOD8} (Panel A). Whether \textit{ENOD8} is fused with MBP or not, it does not have a detectable esterase activity; only the porcine esterase control shows esterase activity (Panel B).
3.9). The gel lanes containing the MBP-ENOD8 factor Xa partial digests did not show any staining at all, for neither the MBP-ENOD8 fusion nor the ENOD8 protein alone.

3.3.8 Antisera Raised against the MBP-ENOD8 Fusion Protein

Milligrams of soluble full ENOD8 protein were purified to raise an antisera for detection of the ENOD8 protein in nodule tissue (Chapter 5). Several liters of the E. coli MBP-ENOD8 recombinant strain were grown and induced with IPTG. Bacterial biomass was recovered by centrifugation and subsequently lyzed with a French press. The ENOD8-MBP fusion was purified from the supernatant lyzate by affinity chromatograpy with amylose resin (3.3.5). Quality and concentration of the purified MBP-ENOD8 fractions were verified by SDS-PAGE and Bradford protein assay, respectively. The MBP-ENOD8 fractions were given to Bio-Synthesis (Lewisville, TX) for immunization of two rabbits. Preimmune sera and antisera from both rabbits were tested by western blot before using either of them for nodule tissue immunostaining (Chapter 5).

3.4 Nicotiana benthamiana Expression System

3.4.1 Expression System Characteristics

Host-specific post-translational modifications may be vital for a protein to be functional (Twyman et al., 2003; Abranches et al., 2005; Brooks, 2006). For an ENOD8 recombinant protein to be active, it may need post-translational modifications, close to the ones of Medicago. Indeed, ENOD8 may have disulfide bonds, and does have a glycosylated pattern (Dickstein et al., 1993; Pringle and Dickstein, 2004). E. coli or
*Pichia pastoris* may not have the post-translational pathways that would make the *ENOD8* protein biochemically active. *N. benthamiana* is a plant and thus would be expected to contain plant-specific post-translational modification systems. The *N. benthamiana* expression system that we chose does not require making permanent transgenic plants and reduces the post-transcriptional gene silencing of the foreign gene (Krebitz et al., 2003; Voinnet et al., 2003). The leaves of *N. benthamiana* are co-infected with *A. tumefaciens* strains carrying the *ENOD8* expression construct or the p19 expression construct. The p19 protein, from the tomato bushy stunt virus (TBSV), is a viral suppressor of post-transcriptional gene silencing (PTGS) in wild-type *Nicotiana benthamiana* (Voinnet et al., 2003).

Dr. Dickstein made two constructs with the *M. truncatula ENOD8* cDNA (GenBank AF064775) under the control of the strong constitutive *CaMV35S* promoter. One construct had the *ENOD8* cDNA sequence and the other construct contained the *ENOD8* cDNA with a 6-His tag tail for purification of the recombinant protein with a nickel column. Dr. Neogi chose the AGL1 and C58C1 *A. tumefaciens* strains for their efficiency of transformation. AGL1 carries the hypervirulent, attenuated tumor–inducing plasmid pTiBo542 from which T–region DNA sequences have been precisely deleted, allowing optimal DNA transformation of many dicotyledonous plants (Lazo et al., 1991). C58C1 was also reported for its efficiency of transformation (Deblaere et al., 1985). *A. tumefaciens* strains were grown for subsequent infiltration in *N. benthamiana leaves*. 
3.4.2 Validation of the *N. benthamiana* Expression System with the Green Fluorescent Protein

The efficiency of the *N. benthamina* expression system was assessed first by surveying leaves of *N. benthamiana*, transformed to express the Green Fluorescent Protein (GFP) (Haseloff and Siemering, 2006). *N. benthamiana* leaves were co-infiltrated with two *A. tumefaciens* strains, one carrying the p19 construct and an other one carrying the GFP construct. Transformed leaves were observed with a fluorescence stereomicroscope. GFP fluorescence was stronger in leaves, which had been co-infiltrated with the *A. tumefaciens* strain carrying the GFP construct or the p19 construct, as compared to leaves only infiltrated with the *A. tumefaciens* strain carrying the GFP construct. In both cases, the fluorescence area was strictly limited to the area that had been infiltrated with the *A. tumefaciens* cultures.

3.4.3 *ENOD8* Expression in the *N. benthamiana* Expression System

When leaves of *N. benthamina* are co-infiltrated with *A. tumefaciens* strains carrying the *ENOD8* expression construct, or the p19 construct, *ENOD8* expression is detected (Figure 3.10). More *ENOD8* protein was present in leaves, which had been infiltrated with the p19 construct as compared to leaves, which had been infiltrated with the *ENOD8* construct alone. The maximum amount of *ENOD8* protein could be recovered at day 2 after infection of *N. benthamiana* leaves with *A. tumefaciens* cultures. Leaves infected with the AGL1 *A. tumefaciens* strain produced more *ENOD8* proteins than the leaves infected with the C58C1 *A. tumefaciens* strain. The size of the
Figure 3.10 ENOD8 protein expression in N. benthamiana leaves analyzed by western blot. ‘a’, ‘b’, ‘c’ lanes: soluble protein fractions from plant independently transformed with the CaMV35S-ENOD8 expression vector. ‘d’ lane: soluble protein fraction from leaves not-transformed with the CaMV35S-ENOD8 expression vector. ‘e’ lane: nodule supernatant as a positive control for ENOD8 protein size. Recombinant ENOD8 protein is detected in the soluble leaf fraction of the three recombinant lines tested.
recombinant ENOD8 proteins was slightly less than the size of the native ENOD8 protein (52kDa) from nodule soluble extract. This could be due to differences in post-translational modifications between N. benthamiana and M. truncatula, such as glycosylation. Because one of the recombinant ENOD8 protein synthetized in N. benthamiana is fused with to a His-tag, recombinant ENOD8 recovery could be done by purification with a nickel column.
4.1 Introduction

Analysis of the ENOD8 sequences suggested that ENOD8 is a secreted protein expressed early during nodulation (Dickstein et al., 2002). Initially M. truncatula roots had been transformed with an A. rhizogenes strain carrying a construct with the GUS gene, whose expression was driven by the promoter of ENOD8.1 (nt7160-10554 of GenBank AF463407). ENOD8 expression was detected in zone III, and possibly II and interzone II-III (R. Dickstein, K. Wilson; unpublished data). The stele of A. rhizogenes-transformed roots stained, which could be a stress response (Dr. D. Barker, personal communication). To avoid non-specific GUS staining, permanent ENOD8-promoter M. truncatula transgenic lines were made using A. tumefaciens (R. Dickstein, P. Neogi; unpublished data). Localization of the ENOD8 promoter GUS expression in developing and mature nodules was confirmed by using T1 progeny from T0 ENOD8 promoter GUS M. truncatula plants from transformation line #10, which had been selected based on its consistent GUS staining pattern in mature nodules of M. truncatula (P. Neogi, unpublished data). Results show that ENOD8 is expressed as early as 5 dpi and persists in mature nodules, where they are detectable from the proximal part of interzone II-III to the proximal part of the nitrogen-fixing (zone III).
4.2 ENOD8 Expression in Mature Nodules

ENOD8 promoter GUS plants from the 2HA transgenic line #10 were grown in aeroponic chamber in a nitrogen-free media mist. After inoculation with S. meliloti, we waited 2-3 weeks for plants to grow mature root nodules. Nodules from different plants were stained. GUS staining was visible in mature nodules from the proximal part of interzone II-III throughout the nitrogen-fixing zone, as indicated by co-localization experiment with iodine staining (Figure 4.1). In the distal part of interzone II-III, GUS expression is very diffuse and looks to be limited to a few cells. From the proximal end of the subsequent zone III, more GUS staining is visible. Several islands of ENOD8 expression can be seen. Then, from the lower distal third of zone III to the proximal end of zone III, GUS staining rapidly increases to much high level of intensity. In this area of zone III, almost all cells exhibit strong GUS staining, indicating that the ENOD8 promoter is active. A sub-population of cells stains very strongly, while another sub-population does not seem to stain at all. The intensity of GUS expression in zone III could be linked to cell invasion by rhizobia: only invaded cells might be staining. With respect to invaded cells, zone III can be subdivided in two intermediate zones, characterized by the bacteroids developmental stage: type 4 and type 5 (Vasse et al., 1990). Type 4 bacteroids were enriched in ribosomes and polysomes, whereas type 5 bacteroids did not have visible ribosomes. Nitrogenase activity is strongly associated with type 4 bacteroids. Nitrogen reduction was undetectable in type 5 bacteroids, as shown by acetylene reduction assay on mature M. sativa nodules (Vasse et al., 1990). The sustained increase in ENOD8 expression could be connected to the changes in
Figure 4.1 \textit{ENOD8} promoter GUS expression localization in a mature nodule. GUS staining is visible just below the interzone II-III (IZ II-III), which is revealed by iodine staining. A few cells stain lightly in the distal end of zone III (white double arrow). Staining intensity rises dramatically in the proximal end of zone III. The onset of \textit{ENOD8} expression coincides with major changes for nodule cells: formation of the symbiosome, bacteroid differentiation throughout zone III. (I: nodule apical meristem)
expression levels of nitrogenase of zone III bacteroids. The intensity of ENOD8 expression does not diminish once it reached its apparent maximum in zone III. Expression is strong in the nitrogen-fixing area of zone III.

4.3 Time-Course of ENOD8 Expression

A time-course for ENOD8 expression was undertaken with the #10 line transgenic 2HA M. truncatula plants, which were transformed with an ENOD8 promoter GUS construct. At 5 dpi, ENOD8 expression is detectable in nodules, which are in the process of emerging from the root cortex (Figure 4.2). Expression is localized in a few discrete areas at the sides of the nodule, toward its proximal end. This result is consistent with northern blot analysis time-course, which had previously shown that the ENOD8.1 gene (GenBank AF463407 nt10554-12564) is expressed 5 days post-inoculation (dpi), after inoculation with S. meliloti (Dickstein et al., 2002). At 8 dpi, ENOD8 expression is distributed unevenly in the proximal two-third of the nodule. The increase of intensity of expression by 8dpi is consistent with the increase of ENOD8.1 mRNA detected by northern blot analysis (Dickstein et al., 2002). In a mature GUS-stained nodule, at 15 dpi, ENOD8 promoter driven expression divides the nodule in two parts. The top third apical part is completely devoid of ENOD8 expression: from the apical meristem (zone I) and include the infection and early symbiosome formation tissues (zone II); interzone II-III was visualized by iodine staining). ENOD8 expression stains intensely and evenly from the proximal part of the interzone II-III toward the proximal end of the nodule. The difference in GUS staining between the mature nodules
Figure 4.2 Time-course of ENOD8 expression in ENOD8 promoter GUS nodules. Time points were: 5 dpi, 8 dpi, and 15 dpi. Panel A: at 5 dpi, faint blue GUS staining (arrowheads) is visible, indicating that ENOD8 expression has started. Panel B: at 8 dpi, ENOD8 expression is distributed unevenly in the proximal two-third of the nodule area. Panel C: at 15 dpi ('1'), ENOD8 is expressed throughout the proximal part of the nodule. Iodine staining ('2') of a section serial to the GUS-stained section ('1') indicates that ENOD8 expression starts approximately from the proximal part of the interzone II-III, which is visualized as a black band by iodine staining ('IZ II-III'). ENOD8 is expressed early during nodulation; its expression persists and increases in mature nodules. (15 dpi data: staining, sectioning, and imaging done by Dr. Purnima Neogi, University of North Texas)
shown in Figure 4.1 and in Figure 4.2 is likely to be due differences in nodule shape, precise position of the sections in the whole nodules, and slight difference in the length of incubation in GUS staining solution.

The persistence of ENOD8 expression in mature nodules is consistent with previous northern blot analysis data (Dickstein et al., 2002). The increase and widespread ENOD8 expression in the proximal half of the nodule from 8 to 15 dpi is nevertheless intriguing. The main function of this area of the nodule is the reduction of atmospheric nitrogen. Previous studies supported the hypothesis that ENOD8 is involved in early events of nodulation and organogenesis of nodules (Dickstein et al., 1991; Dickstein et al., 1993). GUS expression results suggest that ENOD8 may have an additional role in mature nodules.
CHAPTER 5
IMMUNOLOCALIZATION OF THE ENOD8 PROTEINS IN NODULES

5.1 Introduction

Results of the ENOD8 promoter GUS experiment indicated that ENOD8 expression starts in proximal end of the interzone II-III (Chapter 4). ENOD8 is expressed in all tested the zones proximal to the interzone II-III. Localization of the ENOD8 protein at the tissue level by probing with polyclonal ENOD8 antisera was undertaken to confirm the ENOD8 promoter GUS results. Subsequently, experiments to localize the ENOD8 protein at the cell level by immunofluorescence microscopy were carried out. Results showed that the ENOD8 protein is associated with the symbiosome membrane and/or the symbiosome space.

5.2 Anti-Whole ENOD8 Protein Antiserum

The MBP-ENOD8 protein could be purified in quantities sufficient for raising a anti-whole ENOD8 protein antisera (Chapter 3). Previously, an antisera had been raised by injecting rabbits with the M. sativa ENOD8 oligopeptide conjugated to the keyhole limpet hemocyanin (KLH) by Bio-Synthesis (Lewisville, TX) and was shown to specifically recognize the ENOD8 protein from both M. sativa and M. truncatula by western blot (Dickstein et al., 2002). Subsequent tests were aimed at using the antisera to immunolocalize the ENOD8 protein in nodule tissues. The antisera and its corresponding preimmune sera did not have significantly different staining patterns on nodule sections (Dr. Dickstein, personal communication).
Two antisera from two MBP-\textit{ENOD8} immunized rabbits were tested by western blot for their ability to detect specifically the \textit{ENOD8} protein from purified protein nodule supernatant donated by Dr. Janine Sherrier, university of Delaware (Figure 5.1). The MBP-\textit{ENOD8} antisera and a previously raised anti-\textit{ENOD8} oligopeptide antisera (Dickstein et al., 2002) both detected the \textit{ENOD8} protein, at the same 1:10,000 dilution in blocking buffer. Rabbits were not exposed to \textit{ENOD8} proteins prior to being immunized with the MBP-\textit{ENOD8} protein: 1:1,000 dilution in blocking buffer of the preimmune sera from both rabbits did not result in detection of the \textit{ENOD8} protein. Subsequently, the specificity of one MBP-\textit{ENOD8} antisera was verified by immunodepletion. The antisera was incubated with the purified MBP-\textit{ENOD8} protein before probing, which resulted in its inability to detect \textit{ENOD8} protein on a western blot. Thus the anti MBP-\textit{ENOD8} antisera specifically detects the \textit{ENOD8} protein.

5.3 Tissue Preparation for Staining with the MBP-\textit{ENOD8} Antisera

5.3.1 Formaldehyde Fixation

Immunolocalization of \textit{ENOD8} protein in nodules was undertaken using the MBP-\textit{ENOD8} antisera. The nodules were fixed with formaldehyde under a variety of conditions. Concentrations of formaldehyde were 1 or 3%. Nodules were left for fixation at room temperature or at 4°C for 30 min or 15-17 hours (Figure 5.2). Detection was performed using secondary antibodies conjugated with alkaline phosphatase. Nodule sections were incubated with preimmune serum, or with the antisera: there was little difference in the resulting staining patterns. Results were not very consistent between
Figure 5.1 Western blot validation for the MBP-ENOD8 antisera. Detection was performed with alkaline phosphatase-conjugated secondary antibody. Purified protein nodule supernatant (donated by Dr. Janine Sherrier) was run in all lanes of SDS-PAGE gels. The corresponding blots were cut in strips, which were immunoprobed in various conditions. Two MBP-ENOD8 antisera from different rabbits ('83’, ‘84’) were tested. Panel A: both MBP-ENOD8 antisera (‘AS-83’, ‘AS-84’) detected the ENOD8 protein as did the anti-ENOD8 oligopeptide antisera (’524’). Each of the preimmune sera (‘PI-83’, ‘PI-84’) did not detect ENOD8 protein indicating that rabbits were not exposed to ENOD8 proteins prior to being immunized with the MBP-ENOD8 protein. Panel B: specificity of the AS-84 MBP-ENOD8 antisera (‘AS-84’) verified by immunodepletion. AS-84 was incubated with the purified MBP-ENOD8 protein before probing. In lane ‘AS-84 ImD’, no ENOD8 protein was detected. AS-84 was effectively immunodepleted by MBP-ENOD8: it specifically binds to the ENOD8 protein. The anti-ENOD8 oligopeptide antisera was also tested by immunodepletion with the MBP-ENOD8 protein (’524 ImD’). 524 could not subsequently detect ENOD8 protein confirming that the MBP-ENOD8 protein specifically binds to ENOD8 antibodies.
Figure 5.2 Immunostaining for detection of ENOD8 in nodule sections. Tissues were fixed with 3% formaldehyde for 30 minutes at room temperature. Panel A: nodule section was incubated with the MBP-ENOD8 antisera. Panel B: nodule section was incubated with preimmune sera. In the antisera stained section, and in the preimmune sera stained section, isolated cells stain through most of the nodule (black arrowheads). The intensity of staining is stronger for the antisera staining than for the preimmune staining. In both case, the pattern of cells staining in the nodule is very similar.
experimental replicates. From working with the anti-ENOD8 oligopeptide antisera, Dr. Dickstein hypothesized that the fixation steps and the paraffin or plastic embedding caused the loss of the antigenic epitope normally recognized by the ENOD8 antisera. Cross-linking fixative agents like formaldehyde might have hampered the MBP-ENOD8 antisera staining abilities. Immunomicroscopy protocols emphasize the importance of the fixation protocols with respect to maintaining the antigenicity of the protein to be detected and with respect to limiting fixation-induced fluorescence (Pearse, 1980; Bullock and Petrusz, 1982; Mounsey et al., 1999; Ruzin, 1999). It remained to find the appropriate tissue treatment for enhancing the immunodetection of the ENOD8 protein antigen in nodules.

5.3.2 Organic Solvent Fixation

Coagulative fixatives (ethanol, methanol and acetone) were shown to be possible alternate or complementary components of tissue fixation (Sainte-Marie, 1962; Bullock and Petrusz, 1982; Brandtzaeg, 1985). Subsequently, acetone, ethanol and methanol were tested on nodules not fixed with formaldehyde to test for improved ENOD8 staining pattern. Nodules were fixed in cold 96% ethanol, and sectioned with a vibratome. Detection was performed using a secondary antibody conjugated with alkaline phosphatase. Sections incubated with the MBP-ENOD8 antisera showed alkaline phosphatase staining limited to the proximal part of the nodule, accounting for two-thirds of its size (Figure 5.3). In this area, stained cells were completely purple due to alkaline phosphatase activity of the secondary antibody (JIR, West Grove, PA), and
Figure 5.3 ENOD8 immunolocalization in a mature nodule fixed with 96% ethanol. Detection was done by alkaline phosphatase staining. Panel A: a section was incubated with the MBP-ENOD8 antisera. The cytoplasm of some cells stains purple indicating the presence of ENOD8 proteins (see example arrowheads). Panel B: section incubated with the preimmune sera: no cells stain purple. Therefore the staining visible in antisera-incubated section is not due to ENOD8 antibodies which would be present in the sera of animals prior to their immunization with the MBP-ENOD8 protein. ENOD8 stains groups of cells and individual cells in the proximal two-third of the nodule. This area corresponds approximately to tissues where the rhizobia have been internalized in nodule plant cells. As the staining is limited to the cytoplasm of large cells, it is possible that ENOD8 localizes only in invaded cells.
larger than the other cells which did not stain. No staining was visible in nodule sections, which were incubated with the preimmune sera. The ENOD8 protein could be localized in invaded cells from the nitrogen-fixing zone of the nodule, as suggested previously by the ENOD8-GUS experiment results (Chapter 4). The fixation of nodules with 100% methanol at 4°C overnight very much improved the intensity of the staining pattern (Figure 5.4). The staining of nodule sections fixed with absolute acetone also yielded a similar staining pattern, but the patterns obtained were fainter than the immunostaining patterns from ethanol- or methanol-fixed nodules.

Even though the use of methanol fixation showed that the MBP-ENOD8 antisera could detect the ENOD8 protein in situ, not all the large cells within the assumed nitrogen-fixing zone were stained. Also there were significant variations in staining between cells being at the same level in a nodule section: a cell which displayed some staining may have a transversal neighbor that does not stain at all, even though the two cells look very similar. This difference in staining could be due to the absence of ENOD8 in cells looking identical to neighboring cells, which stain positively for ENOD8. Alternatively, it may be that there is a problem with accessibility of the tissue to the antibody. Sections might have been too thick (vibratome sections were 50μm or more) for the primary and secondary antibodies to reach all cells. For example, the cells, which are in contact with the glass slide can only be stained by the antibodies diffusing from the solution through the other cell layers above them.
Figure 5.4 ENOD8 immunolocalization in a mature nodule fixed with 100% methanol. Detection was done with alkaline phosphatase. Panel A: section incubated with the MBP-ENOD8 antisera. Panel B: section incubated with the preimmune sera. ENOD8 staining pattern is similar to the staining pattern seen in Figure 5.2, only the staining intensity is far greater: cells positive for ENOD8 stains in black. Methanol fixation greatly improves detection of the ENOD8 protein in nodule tissues.
5.4 Immunostaining with the Anti-ENOD8 Oligopeptide Antisera

The beneficial effect of organic solvent treatment of the nodules on the detection ability of the MBP-ENOD8 antisera led us to hypothesize that the anti-ENOD8 oligopeptide antisera may work on methanol-fixed nodule sections. Thus we tried the anti-ENOD8 oligopeptide antisera on nodule sections, following the fixation steps presented in 5.3.2. Both antisera were diluted 1:500 in blocking buffer. Detection was performed using a secondary antibody conjugated with FITC. Observations were done with an epifluorescence microscope. Indeed, the staining pattern with the anti-ENOD8 oligopeptide antisera was identical to the staining pattern with the of ENOD8-MBP antisera at the tissue level on nodule sections, which had been fixed overnight at 4°C in 100% methanol (Figure 5.5). The fact that the staining pattern of nodules was identical when either antisera was used strongly suggested that bona fide ENOD8 protein was the antigen recognized in both cases on nodule tissue fixed by treatment with 100% methanol at 4°C.

5.5 Localization of the ENOD8 Protein at the Tissue Level

5.5.1 Purification of Functional and Specific ENOD8 Antibodies by Affinity Chromatography

Once it was established that either of the two ENOD8 antisera gave a strong signal in situ, it was important to show unambiguously that the antisera did contain antibodies that bind specifically to ENOD8 protein and not other antigens. It was more straightforward to purify ENOD8 antibodies from the anti-ENOD8 oligopeptide antisera
Figure 5.5 Staining pattern for the two ENOD8 antisera is similar on nodule sections. Observations were done by epifluorescence microscopy. ENOD8 is detected by FITC fluorescence emission (green signa). Panel A: sections stained with the anti-ENOD8 antisera oligopeptide antibody (‘AS’) or the corresponding preimmune sera (‘PI’). Panel B: section stained with the MBP-ENOD8 antisera (‘AS’) or the corresponding preimmune sera (‘PI’). Both antisera show the same staining pattern: large cells stain within most of the nodule body, except for its distal end. The antisera oligopeptide antibody staining is a little more intense than the staining with the MBP-ENOD8 antisera. Preimmune stained sections shows very little signal, especially for the preimmune sera corresponding to the anti-ENOD8 oligopeptide antisera.
than to purify them from the MBP-ENOD8 antisera. It was decided to make an affinity chromatography column from the commercially available manufacturer (Pierce, Rockford, IL) with the ENOD8 oligopeptide conjugated to beads. The coupling of the ENOD8 peptide (SynPep, Dublin, CA) was carried as instructed by the SulfoLink® Kit. The beads contain an iodoacetyl group, which can react with the free sulfhydryls from cysteine residues. The ENOD8 peptide does have a cysteine residue. 1.74 mg of ENOD8 peptide was used for conjugation on the beads. By measuring the A$_{280}$ of the ENOD8 peptide solution and fractions eluted from the column, I estimated that 0.22 mg of ENOD8 peptide had been conjugated on the beads. Purification of specific ENOD8 antibodies could have been done too from the MBP-ENOD8 antisera. But by purifying antibodies from the MBP-ENOD8 antisera, using a affinity chromatography column cross-linked with the ENOD8 oligopeptide CKNPSTRITWDGTHYTEAA, the ENOD8 antibodies recognizing an other ENOD8 epitope than the ENOD8 peptide would have been lost. Elimination of anti-MBP antibodies from the MBP-ENOD8 antisera could have been done with an affinity column whose beads are conjugated with MBP. But it might have been necessary to add an additional step to eliminate other non-ENOD8 antibodies from the antibody solution. The anti-ENOD8 oligopeptide antisera was incubated in the SulfoLink® affinity chromatography column, whose beads had been conjugated with the ENOD8 peptide. After several column washes to remove unbound or loosely bound molecules, the ENOD8 antibodies were eluted from the column by applying in the column a solution of 0.2 M glycine pH2.0. ENOD8 antibody fractions were collected directly in Eppendorf containing 1 M Tris pH9.2, to prevent damage to purified
antibodies, which could be caused by acidic conditions. The activity of the affinity-purified _ENOD8_ antibody fraction was validated: native and recombinant _ENOD8_ proteins were detected on western dot blots (Figure 5.7). Immunostaining with the affinity-purified _ENOD8_ antibodies of nodule sections fixed with methanol only, resulted in staining patterns identical to the ones observed previously when using the anti- _ENOD8_ oligopeptide antisera or the _ENOD8_-MBP antisera. The affinity-purified _ENOD8_ antibody was immunodepleted by incubation with the _ENOD8_ oligopeptide to test for its specificity toward the _ENOD8_ protein in nodule tissues (Figure 5.6). A nodule section stained with the preimmune sera did not show any immunostaining signal. A nodule section stained with the immunodepleted antibodies showed complete loss of staining except for a few layer cells at the periphery of the nodules, outside of the invaded zone. A nodule section stained with the affinity-purified _ENOD8_ antibody gave an immunostaining pattern identical to the one observed before with the anti- _ENOD8_ oligopeptide antibody used on nodule section (Figure 5.5). Immunodepletion of the affinity-purified antibody by dot blot western gave the same results (Figure 5.7).

Several Immobilon-P (Millipore, Bedford, MA) membranes were spotted with 2 μg of the _ENOD8_ peptide. Each membrane was then incubated with the anti- _ENOD8_ oligopeptide antisera, or the affinity-purified antibody incubated with the _ENOD8_ peptide or not. Antisera and purified antibody revealed the _ENOD8_ peptide spot on the membranes only when they had not been preincubated with the _ENOD8_ peptide. The absence of signal from nodule sections incubated with preimmune sera or incubated only with the
Figure 5.6 Immunodepletion of the affinity-purified ENOD8 antibody on nodule section. The anti-ENOD8 oligopeptide affinity-purified antibody was depleted with the ENOD8 peptide. All sections were incubated with the SYTO-13 green fluorescent dye to visualize the plant cell nuclei and the bacteroids (‘A.2’, ‘B.2’, ‘C.2’). Immunostaining signal is visualized by Cy5 fluorescence (‘A.1’, ‘B.1’, ‘C.1’). ‘A.3’, ‘B.3’, ‘C.3’: merge of the SYTO-13 and Cy5 signals for each panel. Panel A: section was stained with immunodepleted affinity-purified ENOD8 antibody. Panel B: control section stained with the affinity-purified ENOD8 antibody. Panel C: control section stained with the preimmune sera. When the affinity-purified ENOD8 antibody is incubated with the ENOD8 antibody prior incubation with a nodule section, there is complete loss of binding abilities by the antibody (‘A.1’). A control section incubated with the non-depleted affinity-purified ENOD8 antibody shows the expected staining pattern (‘B.1’), as seen in Figure 5.. A control section incubated with the preimmune sera shows no Cy5 signal (‘C.1’). The affinity-purified ENOD8 antibody binds specifically to antigens in the nodule tissues. Bar = 1 mm
Figure 5.7 Immunodepletion test of the affinity-purified ENOD8 antibody by dot western. Each membrane were spotted with 2 µg of ENOD8 peptide. Panel A: the non-immunodepleted affinity-purified antibody does recognize the ENOD8 peptide: purple stain on membrane (‘A.1’); whereas the affinity-purified antibody, which was incubated with the ENOD8 peptide does not stain the membrane (‘A.2’). Panel B: the anti-ENOD8 oligopeptide antisera also does not bind to the ENOD8 peptide on the membrane after its incubation with the ENOD8 peptide (‘B.2’), and binds to it when not pre-incubated with the peptide. Panel C: the preimmune sera does not contain any antibody which can recognize the ENOD8 peptide.
secondary antibodies was very consistent in many trials at low and high resolution. We used blocking buffer in subsequent experiments, as a negative control for staining.

5.5.2 Localization of the ENOD8 Protein in Relation to Nodule Zones

To localize the ENOD8 protein in nodule tissues we proceeded to use the same histological marker as we used for the ENOD8 promoter GUS localization: the interzone II-III (IZ II-III) as revealed by iodine staining (Chapter 4). We also used a confocal microscope (Perkin Elmer Ultraview ERS spinning disk confocal microscope connected to a Zeiss Axiovert 200M) to eliminate out-of-focus fluorescence, which was encountered with conventional epifluorescence immunomicroscopy and hampered observations of immunostained sections.

The first cells staining for the ENOD8 proteins appear in the proximal part of the IZ II-III. At this level not many cells stain for the ENOD8 protein (Figure 5.8). Further down the IZ II-III, toward the proximal part of the nodule, more and more cells stain for the ENOD8 proteins until the proportion of cells staining for ENOD8 staining looks constant. Cells with ENOD8 protein are enlarged and look like they are invaded cells containing the bacteroids.

5.5.3 The ENOD8 Protein in Invaded Cells

To enhance details of tissue structure, nodules were lightly fixed with 0.5% formaldehyde in 1X phosphate pH 7.0. Nodules were vacuum-infiltrated five times for thirty seconds and left for a maximum 20 min at room temperature. Nodules were then
Figure 5.8 Tissue localization of the *ENOD8* protein in nodule serial sections. Panel A: immunofluorescence detection of *ENOD8* protein. Panel B: iodine staining for detection of the interzone II-III (IZ II-III). From the distal end of the IZ II-III onward, most cells stained for *ENOD8*. The size of the stained cells suggested that they are invaded nodule cells. The ring-shaped staining suggests that *ENOD8* proteins co-localized with the cytoplasm of the cells containing the symbiosomes. Bar = 1 mm
transferred to 100% methanol at 4°C for overnight incubation. Shorter incubation times in methanol correlated with a decrease in staining intensity in nodule tissues. Nodules were rinsed several times in phosphate buffer to eliminate methanol and were embedded in low melting point agarose. Thick sections, 50 μm, were obtained from the embedded nodules with a vibratome. Subsequently, nodule sections were triple-stained with SYTO-13 (Molecular Probes, Oregon, USA), a soluble nucleic acid binding dye, the affinity-purified ENOD8 antibody (1:100 dilution in blocking buffer) and the Histone H1 antibody (1:100 dilution in blocking buffer). SYTO-13 can be detected bacteroid DNA and chromosomal DNA (Haynes et al., 2004). Histone-H1 antibody was used as an antibody diffusion control. The assumption is that the diffusion of the histone-H1 antibody and the ENOD8 antibodies should be fairly similar.

SYTO-13 and ENOD8 signals colocalized in cytoplasm of invaded cells (Figure 5.9). The nuclei of these cells also stained for histone-H1, confirming that antibodies could diffuse throughout these cells. Some invaded cells did stain for the ENOD8 protein but not for histone-H1: the nucleus from these cells may have been lost, or just might be absent from the plane of focus photographed during confocal observations (Perkin Elmer UltraVIEW spinning-disk confocal microscope, University of Texas Southwestern, Dallas, TX).

Experiments at the tissue level supported the hypothesis that the ENOD8 protein localized in bacteroid-invaded nodule cells starting from the upper level of the IZ II-III and then continuing throughout proximal zones of the nodule.
Figure 5.9 Localization of the \textit{ENOD8} protein in nodule tissue by confocal microscopy. I: meristematic zone; II: infection and early symbiosome formation zone; III: nitrogen-fixation zone. The nodule from which the section was obtained did not have a senescence zone. Whole nodule section is a merge of the \textit{ENOD8} (Cy5), histone H1 (TRITC), and DNA (SYTO-13) signals. \textit{ENOD8} signal was detected only in invaded cells and increased greatly in intensity towards the proximal part of the nodule, whereas Histone H1 was present evenly in cells throughout the nodule; showing that antigens are accessible to antibodies. The white arrowhead points to a nodule cell staining for \textit{ENOD8} but not for Histone H1 Bar = 50 µm. Picture of the whole nodule is a composite of four individual 20X pictures. (Inset A: Figure 5.11; Inset B: Figure 5.10)
5.6 Localization of *ENOD8* at the Cell Level

5.6.1 Symbiosome Localization

High magnification observation of a cell of the proximal zone of the nodule was done from the nodule section observed at low magnification, described in Figure 5.9 (5.5.3). SYTO-13 staining appears as a pattern of green rods throughout the cytoplasm of large cells localized in the central nitrogen-fixation zone of the nodule (Figure 5.10). The location of the bacteroid DNA was indicated by SYTO-13 green fluorescence. Bacterial chromosomal DNA is mostly homogeneously distributed within the boundary of the bacteroid cells, therefore showing the full shape of the bacteroids. The *ENOD8* signal appeared to be localized precisely around the bacteroid and surrounded it completely (Figure 5.11). This data indicated that the *ENOD8* protein localized somewhere between the cytoplasmic side of the symbiosome membrane and the bacterial membrane facing the symbiosome space.

5.6.2 Variation of the *ENOD8* Staining Pattern in Invaded Cells

High magnification observation of an invaded cell of the distal zone of the nodule was done from the nodule section observed at low magnification, described in Figure 5.5 (5.5.3). The first cells to be invaded do not show *ENOD8* staining (Figure 5.12). In the nitrogen fixation area (zone III) the *ENOD8* protein wrapped completely around the symbiosome, revealed by staining by SYTO-13 (Figure 5.10). Once *ENOD8* protein was detected in the cytoplasm of invaded cells, its staining seemed to grow more intense toward the proximal tissues of the nodule (Figure 5.9). Overall the older the
Figure 5.10 Localization of the *ENOD8* protein in an invaded nodule cell. Confocal immunofluorescence microscopy: higher magnification of Figure 5.5 inset B. Panel A: detection of DNA from bacteroid and nuclei by SYTO-13 fluorescence. Panel B: *ENOD8* protein detected by Cy5 fluorescence. Panel C: Histone H1 detection with TRITC fluorescence (display color was changed to blue for distinguishing the TRITC signal from the Cy5 signal). Panel D: merge picture of panels A, B, C. Rhizobia cells were visible as green rods in the cytoplasm (‘A’). The *ENOD8* staining pattern had the appearance of red small tubular structures throughout the cytoplasm (‘B’). The anti-histone H1 antibody highlighted the nucleus, indicating that antibodies could diffuse throughout the entire cell (‘C’). The merge picture (‘D’) showed that the *ENOD8* staining pattern was wrapped around the bacteroid. The *ENOD8* protein may be localized in the symbiosome space and/or the symbiosome membrane. Bar = 10 µm.
Figure 5.11 Co-localization of the *ENOD8* protein with the symbiosome. Confocal immunofluorescence microscopy was used to image the symbiosomes. Panel A: Bacteroid DNA is detected with the fluorescent nucleic acid dye SYTO-13. Panel B: *ENOD8* protein is detected by Cy5 fluorescence. Panel C: Merge of the SYTO-13 and Cy-5 signals. The cylindric pattern of *ENOD8* follows closely the bacteroid shape, as delineated by the SYTO-13 signal (white single arrowheads). Optical sectioning by confocal microscopy shows some bacteroids in cross sections, emphasizing the location of *ENOD8* protein tightly wrapped around the symbiosome (white double arrowheads). Bar = 10 µm.
Figure 5.12 Absence of the *ENOD8* protein in an early invaded nodule cell. Confocal immunofluorescence microscopy: higher magnification of Figure 5.5 inset A. Panel A: detection of DNA from bacteroid and nuclei with SYTO-13. Panel B: *ENOD8* protein detected by Cy5 fluorescence. Panel C: Histone H1 detection with TRITC fluorescence (display color was changed to blue for distinguishing it from Cy5 fluorescence emission). Panel D: merge picture of panels A, B, C. Rhizobia cells were visible as green rods in the cytoplasm (‘A’). The *ENOD8* staining pattern was limited to a few scattered dots, which could have represented very small of *ENOD8* protein (‘B’). The anti-histone H1 antibody highlighted the nucleus, indicating that antibodies diffused throughout the entire cell (‘C’). The merge picture showed that the *ENOD8* protein did not colocalize with the bacteroid. In early invaded cells, the *ENOD8* protein did not localize in the symbiosome, but may be in the process of being translocated toward that compartment. Bar = 10 µm.
developmental stage of the nodule tissues, the more ENOD8 protein there was. The morphology and function of the bacteroids also changed along the nodule longitudinal axis: bacteroid differentiation started from the early symbiotic zone to the nitrogen-fixation zone or in the senescent zone in old nodules (Vasse et al., 1990; Hirsch, 1992). There appears to be a correlation in ENOD8 protein accumulation and the differentiation of the bacteroid toward its nitrogen-fixing developmental stage.
CHAPTER 6
RNA INTERFERENCE

6.1 Introduction

Immunolocalization and expression results suggested that the ENOD8 protein was constantly synthesized in the nodule in relation to the presence of rhizobia in the cytoplasm, within the symbiosome. If the ENOD8 protein expression were silenced, the nodulation phenotype of Medicago may change, which could give a hint on the role of ENOD8 protein.

Initial experiments were aimed at silencing ENOD8.1 (GenBank AF463407 nt 10554-12564) expression by RNAi interference on M. truncatula with an A. rhizogenes protocol (R. Dickstein, K. Wilson; unpublished data). Results indicated that roots could be regenerated from both ENOD8-RNAi transformed roots and controls.

I used the A. rhizogenes transformation protocol in an attempt to identify nodulation defects, which could be correlated to the absence of ENOD8 protein. ENOD8-RNAi transgenic lines (R. Dickstein, P. Neogi; unpublished data) were then screened for plants, which had the ENOD8-RNAi cassette and which roots showed a decrease in ENOD8 protein expression.

6.2 A. rhizogenes Transformation

Several attempts were undertaken to examine nodulation phenotype of roots of M. truncatula transformed with A. rhizogenes carrying the ENOD8-RNAi construct. Visual analysis with a dissecting microscope did not find significant phenotypic
difference for nodule formation and for mature nodules between transformed not transformed roots (data not shown). The apparent unchanged nodulation phenotype of ENOD8-RNAi plants could have several causes. The function of the ENOD8 gene and its protein product may be unessential or unobservable in the conditions tested. The absence of functionality of ENOD8 could have its roots in a possible functional redundancy for ENOD8: another gene may have a function similar to ENOD8. More broadly, Medicago could overcome the loss of ENOD8 protein through upregulation and downregulation of other genes. As evidenced in Chapter 5, the ENOD8 protein co-localizes with the symbiosome membrane and symbiosome space, which makes ENOD8 a major protein in the mature nodule. Additionally, ENOD8 promoter GUS expression showed that ENOD8 expression is always on in these areas as well (Chapter 4). The activity of the ENOD8 gene is therefore not a negligible phenomena for Medicago. The A. rhizogenes was useful to test rapidly for assessing phenotypes of ENOD8-RNAi root systems. Additional experiments to investigate the effect of ENOD8 RNA interference were carried on with M. truncatula ENOD8-RNAi transgenic lines, which a more robust system, as compared to the M. truncatula hairy root system.

6.3 Transgenic Lines

6.3.1 ENOD8-RNAi Cassette PCR Screening

We screened by PCR for ENOD8-RNAi transgenic plants, which would carry the T-DNA ENOD8-RNAi cassette in their genome. The presence of the ENOD8 inverted repeats separated by the GUS intron made it possible that a DNA hairpin loop would
form during PCR and prevent the thermopolymerase to duplicate the corresponding sequences. Indeed, this amplification only worked on transgenic control plants, which had been transformed for the T-DNA RNAi cassette without the ENOD8 inverted repeats (Figure 6.1). PCR on genomic DNA from ENOD8-RNAi transgenic plants worked on sequences, which were not included in the hypothesized DNA hairpin loop, for example: sequence near the left border of the T-DNA. To overcome the formation of a DNA hairpin loop during PCR, we digested genomic DNA with a restriction enzyme cutting between the ENOD8 sense sequence and the GUS intron, physically separating the two ENOD8 sequences, which should decrease the probability of formation of double-stranded DNA by ENOD8 sequences from the RNAi cassette. Therefore two PCR reactions were designed to detect the two ENOD8 sequences and the GUS intron (Figure 6.2). Genomic DNA samples from putative ENOD8-RNAi plants were used as templates for both PCR reactions, which gave positive results. Together with the PCR for the left border of the T-DNA, these results indicate that several tested plants should have the entire T-DNA ENOD8-RNAi cassette in their genome.

6.3.2 ENOD8 Proteins Levels in Roots

The level of ENOD8 protein in roots was tested by western blot analysis for plants, which had been shown to be likely to contain the ENOD8-RNAi T-DNA cassette. Transgenic lines had various amounts of ENOD8 protein in their roots. Partial loss of ENOD8 protein was observed in several lines to different degrees. One line did show a complete loss of ENOD8 protein in several plants (Figure 6.3).
Figure 6.1 *ENOD8* inverted repeats may prevent direct detection by PCR. Genomic DNA extracts from a *Medicago* plant transformed with the RNAi control vector ('C'), and from a *Medicago* plant transformed with the *ENOD8*-RNAi vector ('E') were tested. The ‘LB-Ins’ primer pair target sequence was a 333 bp sequence in the T-DNA close to its left border, and outside of the neighboring *ENOD8* sequence. The ‘Cass’ primer pair aimed at amplifying the sequence formed by the two *ENOD8* inverted repeats and by the GUS intron (2230 bp). ‘L’: ladder, size of bands is in bp. Both target sequences are amplified by PCR for the control plant. The T-DNA control sequence has been transferred to in the control plant. For the *ENOD8*-RNAi plant, only the ‘LB-Ins’ primer pair amplifies the expected sequence. The ‘Cass’ primer pair did not amplify the expected 2230 bp sequence. The LB side of the T-DNA corresponds to the extremity of the T-DNA, which is transferred into the plant last during transfection with *A. tumefaciens*. Therefore positive ‘LB-Ins’ amplification indicates that the entire T-DNA was integrated in the plant genome. The formation of a double stranded DNA hairpin due to the *ENOD8* inverted repeats could prevent amplification.
Figure 6.2 Detection of the *ENOD8* inverted repeat sequences and the GUS intron. Genomic DNA of an *ENOD8*-RNAi transgenic *Medicago* was incubated with *Bam* HI to digest the T-DNA sequence between the *ENOD8* sense sequence and the GUS intron sequence. The 'LA' and 'RA' primer pairs were designed to amplify template sequences from the T-DNA, including *ENOD8* sequences: the *ENOD8* sense sequence (600 bp expected PCR product), and the sequence consisting of the GUS intron and the *ENOD8* antisense sequence (867 bp expected PCR product), respectively. Both primer pair should not be able to amplify native genomic *ENOD8* sequences. 'L': ladder, size of bands is in bp. Both PCRs produced the expected bands indicating that this *ENOD8*-RNAi plant has the *ENOD8* inverted repeat sequences and the GUS intron from the T-DNA *ENOD8*-RNAi cassette.

Figure 6.3 Loss of *ENOD8* protein in three *ENOD8*-RNAi plants. The protein soluble fraction of transgenic plants likely to have the entire *ENOD8*-RNAi cassette (lanes ‘1’, ‘2’, and ‘3’) or the control RNAi cassette (lane ‘C’) were extracted and tested by western blot with the anti-*ENOD8* oligopeptide antisera. ‘W’: lane with soluble protein fraction from a wild-type plant. ‘L’: ladder, band size in kDa. The three *ENOD8*-RNAi plants show complete absence of *ENOD8* protein. The soluble protein fraction from a wild-type plant and a control plant show the *ENOD8* protein. (Each lane of the corresponding SDS-PAGE gels had been loaded with 20 µg of total soluble protein as evaluated with a Bradford assay)
6.3.3 *ENOD8*-RNAi Phenotype

The plants that had the *ENOD8*-RNAi cassette and no detectable *ENOD8* protein displayed nodulated roots. The pattern of nodulation was not obviously different, as compared to nodulation pattern of wild-type plants (Figure 6.4). These plants will be propagated and used for further study to assess the effect of the absence of *ENOD8* protein.
Figure 6.4 Nodulation phenotype of ENOD8-RNAi M. truncatula plants. ENOD8-RNAi plants (‘1’, ‘2’, ‘3’) contain the ENOD8-RNAi T-DNA cassette (Figure 6.2) and do not have detectable amounts of ENOD8 protein (Figure 6.3). ’1’, ’2’, and ’3’ have nodules comparable to the wild type (‘W’) and the RNAi control plant (‘C’). Scale bars = 1 cm.
7.1 The *ENOD8* Gene Family

A *Hind* III 2.7 kb DNA sequence (nt1-2707 of GenBank AF463407) was subcloned, which corresponds to most of intron 1 (nt92023-94820 of GenBank AC139354) of *ENOD8.3* (nt90647-95068 of GenBank AC139354). Intron 1 of *ENOD8.3* is long as compared to the introns 1 of the other *ENOD8* genes. Previous northern analysis performed on *ENOD8.1*, *ENOD8.2*, and *ENOD8.3* had shown that *ENOD8.1* is expressed at high level, that *ENOD8.2* expression level is 50 times lower than that of *ENOD8.1* and that *ENOD8.3* is not expressed in the conditions tested (Dickstein et al., 2002). A survey of EST database did not yield EST sequences, with high homology to the predicted *ENOD8.3* mRNA (Dickstein et al., 2002). The unusually long intron 1 of *ENOD8.3* might prevent its expression.

Phylogenetic analysis showed that *ENOD8.3* is closely related to the *ENOD8.1-.2* phylogenetic group. The phylogenetic grouping of the *ENOD8* genes could be related to their expression levels in nodulation conditions.

The BAC clone survey contributed to identify *ENOD8.4-.6*, which have high homology at the amino acid level with the protein encoded by *ENOD8.1*. For *ENOD8.4-.6*, no corresponding ESTs were identified (Dickstein et al., 2002). Like *ENOD8.3*, these genes may not be expressed in the nodule. Phylogenetically, *ENOD8.4-.6* have diverged very much from *ENOD8.1*. Two additional *ENOD8* genes, *ENOD8.7* and *ENOD8.8* were found on chromosome 7 and chromosome 8 respectively. Both genes have high
homology at the amino acid level with the protein encoded by ENOD8.1. Both ENOD8.7 and ENOD8.8 are closely related and the ENOD8 genes, but have diverged away the most from ENOD8.1, as shown by phylogenetic analysis. The ENOD8.1-.8 genes form a non-homogenous family with respect to expression in nodules. This is in contrast with a study on another gene family coding for 6 calmodulin-like proteins (CaMLs), which cluster within 110kb of the M. truncatula genome (Liu et al., 2006). It was demonstrated by quantitative RT-PCR that the all 6 CaMLs are expressed in root nodules. Thus ENOD8.1-.8 gene family is different from the CaMLs gene family with respect to the expression properties of its members.

7.2 Recombinant ENOD8 Protein

The P. pastoris and the E. coli recombinant systems tested in this study did not yield ENOD8 proteins with the esterase activity observed for the native M. sativa ENOD8 purified from root nodules, as described in another study (Pringle and Dickstein, 2004). First, the expression of the ENOD8 protein in a soluble form was very much dependent on the host organism and the recombinant system. The Pichia pastoris system did not express detectable amounts of ENOD8 protein. The MBP-ENOD8 fusion protein expressed in E. coli was expressed in a soluble form. Still the apparent ratio of soluble versus insoluble MBP-ENOD8 protein was large. Previous attempts for expression of the ENOD8 protein in E. coli had led to its expression. But the ENOD8 protein accumulated in an insoluble form, probably in inclusion bodies (Dickstein et al., 2002). The analysis of the ENOD8 sequence had not shown that ENOD8 was a very hydrophobic protein: in
fact, ENOD8 was expected to be a soluble protein (Dickstein et al., 1993). The ENOD8 protein was only predicted to have a hydrophobic N-terminal region, which could be a potential signal peptide. The recombinant expression of the ENOD8 protein in a soluble form in leaves of *N. benthamiana* was readily detectable by western blot analysis. The *N. benthamiana* ENOD8 recombinant could be used in future experiments to test for the biochemical activity of the ENOD8 protein. A plant recombinant system may have the post-translational machinery necessary for improving the folding of the ENOD8 protein. The presence of 5 potential glycosylation sites was hypothesized for the *M. sativa* ENOD8 protein, predicted from its cDNA sequence (GenBank L18899) (Dickstein et al., 1993). It was also shown that digestion of purified *M. sativa* ENOD8 proteins with PNGase F, an N-glycosidase digesting glycosylated proteins, showed increase motility on SDS-PAGE (Pringle and Dickstein, 2004), suggesting that ENOD8 proteins are indeed glycosylated. Spectrophotometric assays on the MBP-ENOD8 protein did not detect any esterase activity when incubation was carried with the general esterase substrates which were shown to be hydrolyzed by the *M. sativa* ENOD8 protein purified from nodules in a previous study (Pringle and Dickstein, 2004). Thus the ENOD8 protein biochemical activity may require plant-specific post-transcriptional modifications.

7.3 The ENOD8.1 Promoter is Active in Rhizobia-Invaded Zones of the Nodule

GUS expression results show that ENOD8.1 promoter activity is detected in the area of the nodule from the proximal area of the interzone II-III to the proximal area of zone III. The nodules of *M. truncatula* are indeterminate. Plant nodule cells differentiate
along the nodule longitudinal axis in a distal to proximal direction, with the point of
reference being the root. A nodulin gene, which would be only expressed in early
zones, such as zone II, but not in subsequent zones, could have the protein it codes for
localized in zone II and zone III, because nodule cells transition from one nodule zone
to the next, along a differentiation gradient corresponding to the nodule longitudinal
axis. The stability of β-glucuronidase in tissues (Jefferson, 1989) could possibly mean
that the ENOD8 gene expression is restricted to an area of interzone II-III, and that its
protein product is preserved within the nodule cells as they differentiate. Another
possibility suggested by these results is that ENOD8 is expressed in all the tissues
where X-Gluc was seen, indicative of ENOD8 directed transcription.

7.4 Purification of an Affinity Purified ENOD8 Protein and Development of Fixation
Protocol

The specific localization of the ENOD8 protein in nodule tissues required the
purification of ENOD8 antibodies from the anti-ENOD8 oligopeptide antisera. Their
binding, and subsequent elution from a column with beads conjugated with the ENOD8
peptide did not result in loss of their binding activity as shown by western analysis and
in situ detection. Possibly this could lead to make an affinity chromatography column
with beads conjugated with the anti-ENOD8 oligopeptide antibody. Cross-linked nodules
extracts passed through this column could enable the isolation of complex(es) including
ENOD8 and molecules bound to it. Such molecules could be ENOD8 substrates or
regulators of its function.
The development of a specific fixation protocol was developed to detect the ENOD8 protein *in situ*. The anti-ENOD8 oligopeptide antisera had been highly successful as a tool to identify the native ENOD8 protein from *Medicago* nodules and recombinant ENOD8 protein expressed in various organisms, by western analysis on nylon membrane (Dickstein et al., 2002; Pringle and Dickstein, 2004). But the anti-ENOD8 oligopeptide antisera was found to be unable to recognize the ENOD8 protein in nodule sections, in the conditions tested (Dr. Dickstein, personal communication). When the MBP-ENOD8 was first used on nodules section, the same observation was made. Nevertheless, all these two different ENOD8 antisera had in common was the ability to specifically recognize the ENOD8 protein by western analysis on a nylon membrane. The anti-MBP-ENOD8 antisera would have been expected to have a higher probability than the anti-ENOD8 oligopeptide antisera to recognize the ENOD8 protein in nodule tissues. In principle the ENOD8-MBP antisera should contain a collection of antibodies whose epitopes would be directed toward the different paratopes of the ENOD8 protein: the anti-MBP-ENOD8 antisera was raised against the entire ENOD8 protein (tagged with the MBP domain). In contrast, the anti-ENOD8 oligopeptide antisera was raised against a small fragment of the ENOD8 protein, a 19-mer peptide, making it likely to recognize only a limited number of epitopes on the ENOD8 protein. If the epitopes recognized by the anti-ENOD8 oligopeptide antisera are inaccessible in nodule tissues, the anti-MBP-ENOD8 antisera might be able to recognize other paratopes, which would be accessible for binding by antibodies. These observations led me to search for what could be the critical step(s) of an immunolocalization protocol to enable an antibody to recognize an
antigen in situ. The fixation of tissue is recognized as a critical step in an immunostaining experiment (Ruzin, 1999). Formaldehyde and glutaraldehyde containing fixative solutions had been used for immunostaining experiments for ENOD8 detection in nodule tissues. These fixatives are cross-linkers: they are very effective in maintaining the integrity of tissues at the cell level. In effect, such fixatives can enable very precise localization of the ENOD8 antigen in a cell with light microscopy. Formaldehyde and glutaraldehyde do have disadvantages. Because these fixatives are cross-linkers, they may have changed the antigenicity of the ENOD8 protein, rendering it unrecognizable by its cognate antibodies. Cross-linkers may also reduce the permeability of the nodule tissue section, rendering the access of ENOD8 antibodies inside a section very inefficient. Additionally, the use of formaldehyde or glutaraldehyde also increases noise in immunofluorescence microscopy. Organic solvents are used as alternate fixatives: they preserve tissue by coagulation. Their effect on tissue should not result in changing the chemical formula of antigens. Because they have coagulative properties, they may change the conformation of a protein antigen. Both ENOD8 antisera recognized denatured ENOD8 proteins by western analysis: coagulative fixation should not have extensive deleterious effects on ENOD8 protein detection in situ. Indeed, the use of coagulative fixatives immediately returned a significant ENOD8 detection signal on nodule tissue by alkaline phosphatase detection or immunofluorescence detection when using either ENOD8 antisera. Alkaline phosphatase detection interestingly showed that the kind of organic solvent fixation (methanol, ethanol, acetone) resulted in significant variations in the intensity of the staining in
which pattern of localization was not changed. Additionally, it was noted once that
decreasing the incubation of nodules in methanol from overnight to 30 min resulted in
the loss of significant ENOD8 staining pattern in immunofluorescence microscopy. The
beneficial effect of fixation with organic solvents may reside in their specific ability to
increase the accessibility of ENOD8 protein to cognate antibodies. The use of a light
formaldehyde fixation step was found to be useful to prevent DNA aggregation artifacts,
which might have been caused by fixation with organic solvents. The combined use of
formaldehyde and methanol in specific conditions may be the key to recover the in situ
antigenicity of proteins like ENOD8, which are not detectable using fixation protocols
working for a majority of other proteins.

7.5 The ENOD8 Protein Localizes in Rhizobia-Invaded Zones of the Nodule

With the affinity-purified ENOD8 oligopeptide antibody, we could show that the
ENOD8 protein is localized in invaded tissues of the nodules, from the proximal area of
the interzone II-III to the proximal area of zone III. The results of the localization of
the ENOD8 protein are in good agreement with the localization of the activity of the
ENOD8.1 promoter in nodule tissues. There seems to be a gradient of ENOD8 protein
along the longitudinal axis of the nodule. From its inception in interzone II-III, the
staining pattern of ENOD8 seems to continuously increase until zone III. But the
number of symbiosomes in invaded cells most probably remains the same in each
invaded cell: ultrastructural studied of *M. sativa* nodule have shown that bacteroids stop
dividing in proximal zone II (Vasse et al., 1990). This raises the possibility that the
ENOD8 protein keeps being targeted to nodule tissues as they differentiate proximally along the nodule longitudinal axis.

7.6 The ENOD8 Protein Co-Localizes with the Symbiosome

The ENOD8 protein was co-localized around the bacteroid, in the symbiosome membrane and/or symbiosome space, as determined by confocal immunomicroscopy. Additional data agree with this finding: the ENOD8 protein was detected by western blot analysis in purified symbiosome membrane and symbiosome space gradient centrifugation fractions (Catalano et al., 2004) and in preparation. ENOD8 is not found in all the symbiosomes of the nodule. The early symbiosomes formed in the distal part of zone II when rhizobia are released from their infection threads in the cytoplasm of plant nodule cells (Vasse et al., 1990) are devoid of detectable ENOD8 proteins. Only in the proximal part of interzone II-III does the ENOD8 protein starts to be detectable. The ENOD8 protein is found in all subsequent symbiosomes until the proximal end of zone III. The ENOD8 protein is likely to be targeted to the symbiosome compartment once it has formed.

7.7 Suppression of the ENOD8 Protein Expression

The pattern of localization of the ENOD8 protein changes in nodule as the bacteroid undergoes differentiation. Does the ENOD8 protein have a direct influence on the differentiation of the bacteroid? The RNA interference strategy has been used
successfully in studying the function of other nodulins in relation to symbiosome formation (Limpens et al., 2004; Capoen et al., 2005). ENOD8-RNAi transgenic lines were created (Dr. Rebecca Dickstein, Dr. Purnima Neogi, unpublished results). One ENOD8-RNAi transgenic line was shown to have the ENOD8-RNAi T-DNA sequence in its genome. In the corresponding plants, the ENOD8 protein was not detected in its nodulated root system. At the macroscopic level, these ENOD8-RNAi plants did not show an obvious phenotypic difference with wild-type nodules, in the conditions in which they were tested.

7.8 A Dual Role for the ENOD8 Protein in Nodules?

The ENOD8 gene was first identified as a gene expressed early during nodulation, from 5 dpi, and was found in uninvaded nodules (Dickstein et al., 1993). The finding of ENOD8 protein from the proximal part of interzone II-III, to the entire zone III which are more oriented toward preparing for and maintaining the exchange of metabolites between the plant cells and the bacteroids extends the possible role of ENOD8 which was first hypothesized to be related to nodule organogenesis (Dickstein et al., 1993; Peng and Dickstein, 1994). As the ENOD8 protein density tends to increase strongly toward the end of the nodules, it may be related to a role in defense against rhizobia, driving to senesce in zone IV. The ENOD8 gene is encoding for an early nodulin which accumulates and eventually participates in the senescence phenomena for the bacteroid population so that it does not become too dense in nodule tissues. The ENOD8 protein could play a role in balancing the rhizobia population density in
individual nodules. Indeed, the level of ENOD8 transcripts persists after the addition of nitrate in the plant growth media (Dickstein et al., 2002). Once nitrogen fixation has stopped in nodules, ENOD8 may play a defensive role against rhizobia.

7.9 Conclusion on the Function of the ENOD8 Protein

The ENOD8 protein localizes in the symbiosome membrane and symbiosome space, in the immediate vicinity of the bacteroid. The pattern of ENOD8 protein density throughout the nodule invaded zone increases toward the proximal area of zone III. It is hypothesized that the ENOD8 protein may play a role with respect to nodule defense. This possible new function comes in addition to a role of ENOD8 in the organogenesis of nodules (Dickstein et al., 1993; Peng and Dickstein, 1994). Localization of the ENOD8 protein in the symbiosome also strengthens the previously stated hypothesis that the ENOD8 protein may be have a hydrolytic activity on acetylated extracellular compounds of rhizobia (Pringle and Dickstein, 2004). The ENOD8 protein natural substrate could be the rhizobia’s Nod factor, which has acetyl groups or another bacterially produced substrate.
CHAPTER 8

MATERIAL AND METHODS

8.1 Wild-Type Plants and Transgenic Lines

8.1.1 Seed Germination

*Medicago truncatula* wild-types (A17, 2HA), and transgenic seeds (*ENOD8* promoter GUS, *ENOD8*-RNAi) were recovered from dry seedpods using a lab-made milling device. Seeds were transferred in centrifuge tubes for scarification with concentrated H$_2$SO$_4$. Wild-type seeds were treated for 8 min. Transgenic seeds were scarified for only 1.5 min: they were found to be more sensitive to incubation with concentrated H$_2$SO$_4$. Seeds were rinsed at least 8 times in sterile deionized water to remove all traces of H$_2$SO$_4$. Wild-type seeds used for transformation with *A. rhizogenes* were additionally incubated for 1 min with 6% sodium hypochlorite for surface sterilization. Then, in a laminar flow hood, seeds were promptly rinsed with sterile deionized water for a total of 8 rinses. For subsequent imbibition, all seeds were left in sterile deionized water in centrifuge tubes on a horizontal shaker with gentle agitation for a minimum of 5 hours. Every hour, each tube would be drained and refilled with sterile deionized water. After successful imbibition seeds would look swollen. Imbibed seeds were stored at 4°C, or plated immediately for germination. In the laminar flow hood, seeds were plated on Petri dishes lined with Whatman 3MM filter paper (Fisher Scientific, Pittsburgh, PA). Filter papers were soaked with enough sterile deionized water, to keep humidity level high enough in the Petri dish. In some cases, to prevent
evaporation, Petri dishes were sealed with Parafilm®, which was poked in at least 2 areas, under the rim of the Petri dish lid, to allow for gaseous exchanges. Petri dishes were inverted on their lid and left in the dark at room temperature for 24 hours or more. Seedlings were left to germinate until their radicle was at least 0.5 cm long.

8.1.2 Aeroponic Chambers Preparation

Aeroponics chambers had been prepared as follows. The misting electrical motor is always partially submerged in the liquid plant media. To prevent the liquid from entering inside the motor, it was sealed with silicon glue. The silicon glue was chosen for its absence of detectable toxic effects on *Medicago* and rhizobia growth. Aeroponics chambers were run for 24 hours with the motor in the presence of deionized water and sodium hypochlorite to remove bacterial contaminants. The aeroponic chamber and the motor was then rinsed a minimum of three times with deionized water. Each rinse lasted a minimum of three hours. A nitrogen-free media solution was then poured in the aeroponics chamber (Lullien et al., 1987). The lid of an aeroponics chamber is drilled with numerous holes. Saran wrap film covered the lid of aeroponics chamber. The film was punctured for inserting the grown seedlings in the drilled holes. Saran wrap held seedlings tightly around the hypocotyls. Aerial parts would grow upright, while roots would be continuously in contact with the mist of growth media within the aeroponics chamber.
In sterile conditions, germinated *Medicago* seedlings were sectioned at the hypocotyl level. The sectioned area was dipped onto a bacterial lawn of the appropriate *A. rhizogenes* strain. Plants were put on plates containing growth media, which had been solidified by the addition of Phytablend® (CAISSON Laboratories, Inc., www.caissonlabs.com/) was used at 15 g/l. The growth media was supplemented with 5 mM ammonium nitrate and 25 μg/ml filter-sterilized kanamycin to select for regenerated transformed roots. Plates were then wrapped with Parafilm® to keep their atmosphere humid enough. Holes were poked in the Parafilm® under the lid of the rim to allow for gaseous exchanges. Plates were transferred in the growth room and kept in an upright position to maximize chances of regenerated roots growing on the surface of the solid media. During the root regeneration phase, hypocotyls and newly grown roots tended to grow away from the surface of the media. Plates were inspected daily, and plants’ hypocotyls, or regenerated roots were pushed back onto the media surface. Plants with regenerated roots were then transferred to an aeroponics chamber for subsequent inoculation with *S. meliloti*.

8.1.4 *M. truncatula* Transgenic Lines

8.1.4.1 ENOD8 Promoter GUS Lines

The ENOD8 promoter GUS construct was made by Dr. Dickstein, which she described as follows (Dr. Dickstein, personal communication). A pRD022 plasmid was
engineered by cloning the 3.2 kb SpI piece of pCAMBIA2301 into pUC18. This 3.2 kb piece of pCAMBIA2301 contains lacZα, the CaMV 35S promoter, and GUS containing the catalase intron. pRD022 was obtained by selecting for the darker blue colonies on X-Gal-containing media that contain two tandem copies of lacZ. The ENOD8.1 promoter was obtained with flanking Sac I and Nco I restriction sites by PCR from BAC mth1-19n23 (GenBank AF463407) by PCR with the following primers: Sac I-ENOD8p: 5'-GAT CGA GCT CCA CCG GAC CTA TTG ACT AGC – 3’ and ENOD8p-Nco I: 5’-GCA TGC CAT GGA TTT CAT GAA GCA ATA AAG GAA CC – 3’. These primers amplify DNA from nucleotides 7570 to 10551 from GenBank AF463407 (BAC mth1-19n23) that contains 2.98 kb of DNA immediately upstream of the translational start codon of ENOD8.1 (nt7160-10554 of GenBank AF463407). The 2.98 kb PCR fragment was restricted with Sac I and Nco I and ligated to similarly restricted pRD022, creating a translational fusion between the ENOD8.1 upstream sequence and GUS. The PCR manipulations changed the sequence at the translational start of ENOD8.1 from 5’-TTCATGG to 5’-TC TCCATGG. The resulting plasmid was sequenced and subsequently digested with Sac I and BstE II. The 4.23 kb Sac I-BstE II fragment containing the ENOD8.1 promoter fused to GUS was subsequently ligated into pCAMBIA2301, creating pRD027 (Figure 8.1).

Dr. Neogi introduced the constructs in A. tumefaciens and created the M. truncatula transgenic lines. The plant transformation method is described in paragraph 8.1.4.3.
Figure 8.1 *ENOD8* promoter GUS cassette. The GUS expression is driven by the *ENOD8.1* promoter (nt7160-10554 of GenBank AF463407).

Figure 8.2 *ENOD8*-RNAi cassette with PCR primers used for its detection. The *ENOD8* sequence corresponds to 514 nucleotides from the *M. truncatula* cDNA (nt209-722 of GenBank AF064775). For each primer pair the forward primer is shown on top of the RNAi cassette, while the reverse primer is shown below the RNAi cassette. The length of each subsequence in the T-DNA is not drawn to scale. For the four primer pair sequences: ‘f’ stands for the forward primer, ‘r’ stands for the reverse primer. T-DNA is copied from the plasmid carried by *A. tumefaciens*. The single-stranded DNA sequence enters a plant cell, by its left border (‘LB’). The right border (‘RB’) enters the plant cell last. The pMAS2’ promoter drives the expression of the *NPTII* gene, which confers resistance to kanamycin to the transformed plant cell. The two *ENOD8* inverted sequences borders the GUS intron sequence. The constitutive promoter p35S drives the expression of the latter *ENOD8* - GUS - *ENOD8* sequence, which forms an RNA hairpin loop. The ‘Cass’ primer pair was meant to detect a sequence including the *ENOD8* inverse repeats and the GUS sequence. It only amplified a product on the control vector devoid of the *ENOD8* sequences. The ‘LB ins’ primer pair detects a sequence located between the left border and the next *ENOD8* sequence. The ‘LA’ primer pair detects the *ENOD8* sequence closest to the left border. The ‘RA’ primer pair detects a sequence including the GUS sequence and the *ENOD8* sequence closest to the right border.
8.1.4.2 ENOD8 RNAi Lines

The ENOD8 sequence used for making the ENOD8-RNAi construct corresponds to a 514 nucleotides sequence (Figure 8.2) from the M. truncatula cDNA (nt209-722 of GenBank AF064775). The first codon of the sequence corresponds to Arginine 55 in exon 1 of ENOD8.1 (GenBank AAC26810), and the last codon corresponds to threonine 225 at the start of exon 4 of ENOD8.1.

Dr. Neogi introduced the constructs in A. tumefaciens and created the M. truncatula transgenic lines. The plant transformation method is described in paragraph 8.1.4.3.

8.1.4.3 Transformation with A. tumefaciens

2HA (Rose et al., 1999) sterilized seeds were germinated for 2 days. 2-3 weeks old plantlets were used for transformation. The transformation protocol was used as previously described (Chabaud et al., 1996; Rose et al., 1999; Chabaud et al., 2003). Trifoliate leaves were cut from the plants and wounded. Transformation was performed by co-cultivating leaves and A. tumefaciens strains. Co-cultivation of leaves and A. tumefaciens strains lasted for 3 days, in flasks, under gentle agitation in a growth cabinet (25°C, 16/8h photoperiod). Leaf explants where then incubated for three days with hygromycin to eliminate A. tumefaciens. Subsequently, callogenesis was induced on the transformed leaves: leaf explants were laid on solid callogenesis induction media medium with kanamycin and augmentin for three weeks. Embryogenesis of calli was induced by embryogenesis induction media, supplemented with kanamycin and
augmentin for 4-6 weeks. Globular to torpedo-shaped embryoids were obtained. Mature embryoids were then transferred to embryoid development media supplemented with kanamycin and augmentin.

8.1.4.4 ENOD8-RNAi Lines Screening by PCR

Genomic DNA was extracted (8.2.2.) as template for various PCR reactions. 4 pairs of primers were used for PCR screening (Figure 8.2). The ‘Cass’ primer pairs ('Cass' f: 5' - GGC GGT AAG GAT CTG AGC TA - 3', and ‘Cass’ r: 5' - TGT GCG TGG CTT TAT CTG TC - 3') aimed at detecting the core of the T-DNA ENOD8-RNAi cassette: the ENOD8 inverse repeats and the GUS sequence. The ‘LB ins’ primer pair ('LB ins' f: 5' - CCA ACG ATT TGT CGT CAC TG - 3' and ‘LB ins’ r: 5' - GCA GAA CCG GTC AAA CCT AA - 3') amplifies a sequence located between the left border and the adjacent ENOD8 sequence. The ‘LA’ primer pair ('LA' f: 5' - GCAC AAC AGA ATT GAA AGC AAA - 3' and ‘LA’ r: 5' - CGA GCA CCA CCT TCT CCA TA - 3') amplifies the ENOD8 sequence closest to the left border. The ‘RA’ primer pair ('RA' f: 5' - AGA GAT CGC TGA TGG TAT CG - 3' and ‘RA’ r: 3' - TCC AAA ATG ATC TTG CAC CT - 3') detects a sequence including the GUS sequence and the ENOD8 sequence closest to the right border. For the ‘LA’ and ‘RA’ amplification, a BamH I digest of the genomic DNA template was performed (2 µl DNA extract, 13 µl deionized water, 2 µl 10X buffer, 2 µl 10X BSA, 1 µl BamH I (New England Biolabs, Beverly, MA). Digestion was carried out in a Thermal cycler set at 37°C for at least 2 hours. PCR reactions used 1 µl of digest as template. In each PCR tube, the concentration of reagents was as follows: 1X ThermoPol buffer, 0.2 mM of each
8.1.5 Plant Media Solution

Nitrogen-free media solution was prepared from 100X or 1000X stocks of micro- and macroelements. These stock solutions were diluted with sterile deionized water to 1X working concentrations: 0.52 mM K$_2$SO$_4$, 0.25 mM MgSO$_4$, 1 mM CaCl$_2$, 50 μM Na$_2$EDTA, 30 μM H$_3$BO$_3$, 10 μM MnSO$_4$, 0.7 μM ZnSO$_4$, 0.2 μM CuSO$_4$, 1 μM Na$_2$MoO$_4$, 0.04 μM CoCl$_2$, 5 mM potassium phosphate buffer pH 7.0 (1 L of 100X stock was made with 174.16 g of K$_2$HPO$_4$, 136.09 g of KH$_2$PO$_4$). Ferrous chloride and ferrous sulfate salts were added to the media to reach 0.033 μM and 0.024 μM, respectively.

8.1.6 Inoculation with Rhizobia Strains

Seedlings were left to grow for five days in an aeroponics chamber to deplete their nitrogen stocks before inoculation with rhizobia. During that time, the aeroponics
chambers were left in the lab to avoid chance inoculation with rhizobia. *M. truncatula* was inoculated with *S. meliloti* ABS7 (Bekki et al., 1987), or strain Rm2011 (Rosenberg et al., 1981). Rhizobia strains were grown in TY liquid media supplemented with 10 μg/ml tetracycline for *S. meliloti* ABS7 culture. Rhizobia cultures were grown for 1 to 1.5 days at 30°C with gentle agitation. Cultures were centrifuged to remove growth media. Rhizobia pellets were then resuspended in deionized water by vortexing. For inoculation, the aeroponic chambers were transferred to the growth room: resuspended rhizobia solution was poured in the media, at the bottom of the aeroponics chambers.

8.1.7 Growth Room Conditions

Plants were kept in a growth room after inoculation with *S. meliloti*, at 22°C on a 16-h-light and 8-h-dark schedule under Phillips Agro-Lite bulbs (Phillips, Somerset, NJ) at 60 μmol.m⁻².s⁻¹, as previously described (Veereshlingam et al., 2004).

8.2 DNA Isolation

8.2.1 Plasmid Preparation by Alkaline Lysis

*E. coli* strains were grown in LB media supplemented with the appropriate antibiotics for 16 hours at 37°C with vigorous shaking. BAC clones were grown in LB containing chloramphenicol (12.5 μg/ml). Bacterial cells were harvested by centrifugation. Cultures over 500 ml were washed at least once in STE (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Plasmids were extracted based on the alkali
lysis method (Birnboim and Doly, 1979; Sambrook et al., 1989). Bacterial pellets were resuspended in Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). For large extract, freshly prepared lysozyme (10 mg/ml) in 10 mM Tris-HCl pH 8.0, was added to the bacterial suspension. Solution II (0.2 N NaOH - freshly diluted from a 10 N stock, 1% SDS) was then added to the mixture for cell lysis. After gentle homogenization, the mixture was left at room temperature for 5-10 min. Solution III (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of deionized water) was then added to cell lysate mixture to precipitate out chromosomal DNA along with cell debris. The soluble phase was recovered by centrifugation and further purified by a phenol-chloroform extraction step, to eliminate protein contaminants. Plasmids were precipitated at room temperature by addition of 2 volumes of 100% ethanol or 0.6 volume of 100% isopropanol. Plasmids were pelleted by centrifugation and washed with 70% ethanol. DNA pellets were resuspended in TE pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Alternatively, plasmid DNA was extracted with solutions and purification columns from a Qiagen kit (Qiagen, Valencia, CA).

8.2.2 Plant Genomic DNA Extraction with CTAB Buffer

Extraction protocols of genomic DNA from *Medicago* was based on published procedures (Murray and Thompson, 1980; Ausubel, 1987) and protocols developed in other *Medicago* labs. For the purpose of PCR, genomic DNA extraction was performed as follows. One leaflet of a trifoliate leaf, fresh or from a -80°C stock was transferred in a fresh Eppendorf tube. Under liquid nitrogen, the leaflet was grinded with a plastic
pestle (Bel-Art Products) fitting the shape of the bottom of the Eppendorf tube. 700μl of 2X CTAB (2% (w/v) CTAB, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% PVP, 0.2% BME) were added to the grinded tissue. Additional grinding was done with the pestle to disperse the tissue in the extraction buffer. The grinded leaflet in extraction buffer was then incubated for at least 30 min at 65°C in a water bath. 570 μl of chloroform:isoamyl alcohol (24:1) were added to the extract. After shaking the sample to emulsify the chloroform:isoamyl alcohol with the aqueous phase, the sample was centrifuged at 4°C for 5 min at 13,000rpm in a microcentrifuge. Supernatant was transferred in a fresh tube. Pipeting was done carefully to avoid carrying over solvent traces from the inorganic phase, and from the solid interphase debris disk. DNA was precipitated by adding 0.7 volume of 100% isopropanol. Tube was centrifuged at 4°C for 10 min at 13,000 rpm in a microcentrifuge. After removal of the supernatant, the DNA pellet was washed once with 70% ethanol. Traces of ethanol were removed by letting the pellet dry at room temperature for 20 min. DNA was dissolved in 25 μl of TE pH 8.0. For the purpose of Southern blot analysis, the following modifications were implemented for the extraction. Several grams of leaf tissues were collected and grinded with a porcelain pestle and a porcelain mortar, under liquid nitrogen. The powder was never allowed to thaw before it got in contact with the extraction buffer. The elimination of proteins and other contaminants was performed with phenol:chloroform:isoamyl alcohol (25:24:1). RNA was eliminated by incubating the soluble phase with DNase-free RNase to a final concentration of 20 μg/ml at 37°C for 15 min. Elimination of the RNase was performed by a phenol: chloroform:isoamyl
alcohol (25:24:1) extraction step. DNA was precipitated by addition of 0.1 volume of sodium acetate (0.3 M stock, pH 5.2) and 0.6 volume of isopropanol.

8.3 Subcloning of Nt1-2707 of GenBank AF463407

8.3.1 Fragment and Insert Preparation

BAC clone 19N23 was digested to completion with Hind III (New England Biolabs, Beverly, MA). The pBluescript® SK- (Stratagen, La Jolla, CA) plasmid vector, which has a unique Hind III site in its MCS, was also digested with Hind III. Both digests were purified by a phenol:chloroform:isoamylalcohol (25:24:1) extraction step and resuspended in TE pH 8.0. The digested vector DNA concentration was evaluated by running it on an agarose gel, against the uncut vector at various known dilutions. The DNA concentration of the Hind III digest was evaluated by measuring its absorbance with a spectrophotometer at 260nm.

8.3.2 Ligation of Vector and Insert

The cut vector and the BAC19N23 digest were setup for ligation by using 5 different insert to vector ratios (1:1, 1:3, 1:10, 3:1, 10:1). Ligation controls were: cut vector without added insert, in the presence or in the absence of T4 DNA ligase. 10μl ligation reactions were setup with 0.5 μl T4 DNA ligase (New England Biolabs, Beverly, MA), 1 μl 10X ligase buffer (New England Biolabs, Beverly, MA), sterile deionized water,
Cut vector solution, and insert solutions. Ligations were carried out for 16 hours at 16°C in a Techne PHC-3 (Princeton, NJ) thermal cycler.

8.3.3 Transformation of Ligation Products in E. coli Competent Cells

Ligation products were transformed into competent cells according to the manufacturer guidelines with Epicurian Coli® XLII-Blue ultracompetent cells (Stratagen, La Jolla, CA). Briefly, competent cells were taken out of -70°C storage and thawed on ice for 12 min. After gently swirling the cell, 2 μl of β-mercaptoethanol from the Epicurian Coli® XL2-Blue ultracompetent cells kit were added to 100 μl of competent cells on ice. Tubes were swirled gently and incubated on ice for 10 min. Tubes were swirled gently every 2 min. In every competent cells tube, 1 μl of ligation product was added. As transformation controls there were: (1) 1 μl of uncut vector added to one transformation tube, and (2) no DNA added to one transformation tube. All tubes were gently swirled and left to incubate on ice for 30 min. The tubes precisely underwent a 42°C heat-pulse in a water bath for 30 sec. Tubes were immediately put back on ice for 2 min. 0.9 ml of NZY® (10 g/l NZ amine (casein hydrolyzate), 5 g/l yeast extract, 5 g/l NaCl, 12.5 mM MgCl₂, 12.5 mM MgSO₄, 20 mM glucose; pH adjusted to 7.5 with NaOH) was added to each transformation tube. Tubes were then incubated at 37°C for one hour with shaking (225-250 rpm). Plates for transformation mixture were made of LB media supplemented with 100 μg/ml ampicillin; on each plate, a mixture of 0.5 ml deionized sterile water, 50 μl 2% X-Gal dissolved in DMF, and 10 μl of 100mM IPTG was spreaded. Transformation mixtures were plated on the latter plates and incubated
overnight at 37°C. Positive colonies would be expected to be white: their plasmid should have an insert in the MCS preventing the transcription of lacZ, and therefore the hydrolysis of X-gal in a blue insoluble product. White colonies corresponding to the 5 experimental ligations were further screened by colony blot hybridization.

8.4 Southern Blot Analysis

8.4.1 DNA Quantification, Digestion, Agarose Electrophoresis, Gel Recovery

Plasmid or genomic DNA was quantified by measuring absorbance at 260nm with a spectrophotometer. Purity of the DNA extract was evaluated by measuring the A_{260}/A_{280} ratio. All restriction digests were carried with restriction enzymes from New England Biolabs (Beverly, MA). Typically, quality control digests would be carried out in a 20 μl volume. The volume of restriction enzyme never exceeded 10% of the reaction volume, to keep glycerol concentration always less than 5% in the digest volume in order to avoid partial digestion of DNA. BSA addition in the digest, and temperature of incubation (almost always 37°C) was according to New England Biolabs (Beverly, MA) instructions. For small amounts of DNA, incubation time averaged 1 to 1.5 hour. For large digest, digestion time was extended; digestion completion was verified by running aliquots of large digests on agarose gel. DNA fragments needed for radiolabeling were further isolated by electrophoresis on polyacrylamide or agarose gel, depending on their size. Gels were stained by soaking them in 0.5 μg/ml ethidium bromide solution. With a UV transilluminator, the DNA band of interest could be seen, and the corresponding gel
piece containing it cut off the whole gel. Typically, the collected gel pieces were cut with a razor blade in smaller fragments and transferred in a container filled with elution buffer (0.5 M ammonium acetate, 1 mM EDTA – pH 8.0). DNA was left to elute out of the gel medium by diffusion, overnight, at 37°C on a rotating platform. Elution mixture was centrifuged to recover the supernatant from which the DNA fragment would be precipitated by the addition of two volumes of 100% ethanol. After centrifugation, the DNA pellet was resuspended in TE pH 8.0. An aliquot of the solution was run on polyacrylamide or agarose gel to evaluate the quantity of DNA, which had been isolated.

8.4.2 DNA Capillary Transfer onto Nylon Membranes

DNA samples were run on 0.8-1% agarose gel in 1X TBE (0.09 M Tris-borate, 0.002 M EDTA). Gels were soaked in 0.5 μg/ml ethidium bromide solution. DNA was visualized by laying stained gels on a UV transilluminator table. Gel was photographed with a Polaroid camera. Unused areas of the gel were trimmed away. The well area of the gel was kept to make it easier later to overlap the digested pattern of gels and the southern hybridization pattern of exposed X-ray films. All following incubations were done at room temperature. The gel was soaked for 45 min in several volumes of denaturation solution (1.5 M NaCl, 0.5 M NaOH) with gentle agitation; typically there was one solution change after the first 15 min of incubation. A rinse was done with deionized water, before neutralizing the gel by soaking it for 30 min in several volumes of neutralization solution (0.5 M Tris-HCl pH 7.4, 1.5 M NaCl), with one solution change.
for an additional 15 min of incubation. The nylon membrane used for DNA transfer was first floated on a surface until it wetted completely from beneath. The membrane was then inverted in 10X SSC transfer buffer (0.15 M sodium citrate, 1.5 M NaCl – pH 7.0) for at least 5 min. A transfer pyramid was set up to transfer the DNA from the gel onto the nylon membrane by buffer diffusion. A glass plate was put across a tray filled with 10X SSC. A piece of 3MM Whatman paper, which had been soaked in 10X SSC was laid flat across the glass plate for two opposite edges to dip into the 10X SSC solution of the container. At this step, as in any subsequent step when a new layer was added to the pyramid, a glass pipet was rolled on the paper to squeeze out trapped air bubbles, which would interfere later with the flow of buffer and therefore the DNA transfer. The gel was removed from the neutralization solution, inverted and laid on top of the filter paper. Saran wrap was place all around the edges of the gel so that later during DNA transfer, buffer from the container would not flow directly to the paper towels of the top of the pyramid. The nylon membrane soaked with 10X SSC was placed on top of the gel. Two pieces of 3MM Whatman paper soaked with 10X SSC were laid on top of the nylon membrane. A ~ 10 cm high stack of paper towels was placed on top of the pyramid, topped with a glass plate supporting a 0.5 kg weight. Transfer proceeded for 24 hours or less. Wet paper towel were replaced with fresh paper towel as needed. After transfer, the nylon membrane was soaked in 6X SSC for 5 min at room temperature, to remove pieces of agarose, which might be sticking on the membrane. Liquid was drained away from the membrane, which was left to dry flat on a paper towel for at least 30 min at room temperature. DNA was cross-linked on the membrane
by short exposure to UV. The side of the nylon membrane carrying the DNA was laid in UV cross-linking chamber of a *UV Stratalinker* for 12 sec (Stratagen, La Jolla, CA). The membrane was stored in a plastic sheet folder. The transferred gel was stained in a 0.5 μg/ml ethidium bromide solution and photographed when laid on a UV transilluminator, to assess the extent of the transfer.

8.4.3 Hybridization of Radiolabeled Probes to DNA Immobilized on Nylon Membranes

8.4.3.1 Prehybridization of DNA Blot

Blots were first re-wetted in a 6X SSC solution. Blots were then incubated inside a sealed bag with gentle agitation on a horizontal shaker for 1-2 hours at 42°C in prehybridization solution (6X SSC, 5X Denhardt’s reagent, 0.5% SDS, 100 μg/ml denatured and fragmented salmon sperm DNA, 30% formamide for low stringency hybridization conditions or 50% formamide for high stringency hybridization conditions). Approximately 0.2 ml of prehybridization solution was used for each square centimeter of nylon membrane.

8.4.3.2 DNA Labeling with Radioisotopes

DNA fragments or undigested BAC plasmids were labeled by random labeling with the random primed DNA labeling kit (Boehringer Manneheim) or with solutions prepared by Dr. Rebecca Dickstein. First the DNA solution was boiled for 10 min, then quickly chilled on ice for 5 min. dATP, dGTP, dTTP, α-[^32]P-dCTP, hexanucleotides
primers and the Klenow fragment were added to the DNA. The labeling mix was incubated at 37°C for 30 min. Reaction was stopped by addition of EDTA. A 10 cc syringe filled with Sephadex G-50, was used as filtration column to separate the radiolabeled probe from unincorporated α-32P-dCTP. Labeled mixture was loaded on top of the column, which was inserted in a tube, which was briefly centrifuged. An aliquot of the recovered radiolabeled probe, was tested for its activity with a cpm counter, the Bioscan/QC•2000 (Bioscan inc., Washington, DC).

8.4.3.3 Hybridization of DNA Blot with Radialabeled Probes

Radiolabeled probes were denatured by boiling them for 5 min, and chilled rapidly on ice. The denatured probe was added to the bag containing the blot in the prehybridization solution. After resealing, the bag was put back on the rotary shaker at 42°C for 15 hours. Blots were then washed with solutions containing SSC and SDS as follows. Genomic blots probed at high and low stringency were incubated for 30 min at 37°C in 2X SSC, 0.5% SDS. A second incubation was performed for 30 min in 0.2X SSC, 0.5% SDS at 37°C for low stringency blots, and at 60°C for high stringency blots. BAC blots, which were probed at high stringency were washed in 2X SSC, 0.5% SDS at room temperature for 5 min, then washed in 2X SSC, 0.1% SDS at room temperature for 15 min, then washed in 0.1X SSC, 0.5% SDS at 37°C for 45 min, then washed in 0.1X SSC, 0.5% SDS at 68°C for 45 min. The blots which were probed with the Hind III - Sac I fragment (nt2623-2702 of GenBank AF463407), were washed as follows. Blots were incubated in 2X SSC, 0.5% SDS for 5 min at room temperature, then incubated for 15
min at 65°C, without solution change. Afterwards all blots were rinsed in 0.1X SSC.

Blots were laid sandwiched in between Saran wrap film and laid in a cassette. In the dark room, an X-ray film was then laid on top of the blot. To increase the intensity of the signal on the film, an intensifying screen was put on top of it. The film cassette was left at -70°C for various exposure times. X-ray films were developed with an X-ray developer.

8.4.4 Removal of Radiolabeled Probes from DNA Blots

Blots previously hybridized with a radiolabeled probe were stripped as follows. They were washed twice for 15 min at 90°C in 0.1X SSC, 0.5% SDS. Blots were briefly rinsed in 0.1X SSC. Blots were re-exposed with an X-ray film for three days to check that all no radioactive material was left bound on the blot.

8.5 Colony Blot Hybridization with Radiolabeled Probes

Colonies were grown on nylon membrane laying on Petri dishes filled with media supplemented with the adequate antibiotics. The colony-studded membranes were processed by laying them on Whatman 3MM filter papers, which had been soaked with the adequate solutions (Sambrook et al., 1989). Bacterial cells were lysed by exposure to 10% SDS for 3 min. The nylon membranes were then exposed successively to a denaturation solution, a neutralization solution, and a 2X SSC solution for 5 min in each
case. After drying for 30 min, the DNA was crosslinked as described in 8.4.2.
Subsequent hybridizations were carried out as described in 8.4.3.

8.6 Bioinformatics

DNA and protein sequences of interest can be retrieved by querying them by their GenBank number on the NCBI main page (www.ncbi.nlm.nih.gov/) or in the NCBI Entrez search engine (www.ncbi.nlm.nih.gov/gquery/gquery.fcgi). Search of sequences with high homology to a given DNA or protein sequence was done with the BLAST tool from NCBI (www.ncbi.nlm.nih.gov/BLAST/). To look for similarities between two given sequences I used the BLAST2 sequences tool from NCBI: (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The phylogenetic tree was obtained from the ClustalW tool of EMBL-EBI (www.ebi.ac.uk/clustalw/). Sequences were put together in a single FASTA file uploaded to the EMBL-EBI website. Information on BAC sequences were retrieved from the M. truncatula consortium website (www.medicago.org/), by consulting the genome assembly browser (e.g.: chromosome 1, www.medicago.org/genome/contig_viewer.php?chromosome=chr1), by consulting the IMGAG and Fgenesh annotations when available (e.g.: BAC clone AC139354, dna8.genome.ou.edu/cgi-bin/gbrowse/medicago_new/?name=AC139354). For alignment of DNA or protein sequence, I used BioEdit, which can be downloaded at: www.mbio.ncsu.edu/BioEdit/bioedit.html. The NEB cutter website was used to find restriction sites on various DNA sequences (tools.neb.com/NEBcutter2/index.php). After performing a custom digest of given a DNA sequence, the NEB cutter site gave the option of viewing a virtual representation of how the agarose electrophoresis band patterns would look like.
Primer sequences were predicted using the on-line ‘OligoPerfect™ Designer’ tool from Invitrogen (www.invitrogen.com/content.cfm?pageid=9716).

8.7 ENOD8 Recombinant Expression Systems

8.7.1 Pichia pastoris Expression System

8.7.1.1 Engineering of ENOD8 Expression Cassette

The nt100 to 1158 DNA fragment of the M. sativa ENOD8 cDNA (GenBank L18899) was amplified by PCR with the Xho I and Not I primer pairs for cloning in the pPICZαC vector. The Xho I primer (5’ - GCC TCG AGA AGA GAG AGG CTG AAG CAT CAA CAC ATT GTG ATT TTC CT - 3’) restored the Kex2 signal cleavage site for the α-factor secretion signal of S. cerevisiae to be cut off by Pichia pastoris from the recombinant ENOD8 during expression, and engineered a Xho I site at the 5’ end of the PCR product. The Not I primer (5’ – GCG CGG CCG CAT CAC TTT CTA TAA CAT GCC AT – 3’) engineered a Not I site at the 3’ end of the PCR product. 25 μl PCR reactions were setup: 1X polymerase buffer, 0.25 U Vent polymerase (New England Biolabs, Beverly, MA), 2.5 mM for each dNTPs (dTTP, dGTP, dCTP), 0.25 μM for each primer, diluted template (~1,000,000 copies), sterile deionized water. Thermal file was: 2 min at 94°C, followed by 30 cycles with a denaturation step (1 min at 94°C), an annealing step (1 min at 55°C), an extension step (2 min at 73°C). PCR was completed with an 8 min incubation at 73°C to complete the extension of all PCR products.
8.7.1.2 Subcloning in pUC18 of the Customized ENOD8 Fragment

The customized DNA fragment was first subcloned in pUC18 (Sambrook et al., 1989). The PCR product was recovered from a low melt agarose gel (1X TAE) and purified from contaminants by phenol-chloroform extraction. The DNA pellets were resuspended in 1X TM buffer (50 mM Tris-HCl pH 8.0, 15 mM MgCl₂). The purified PCR products were then treated to add a phosphate group at their 5' ends and to fill any nucleotide gaps in order to make them blunt-ended. DNA was incubated at 37°C in 47 µl reaction volume (5 µl of 10X kinase buffer (New England Biolabs, Beverly, MA), 5 µl of 10 mM rATP, 7 µl of 0.25 mM for each dNTPs (dATP, dTTP, dGTP, dCTP), 0.5 µl of 10 U/µl T4 polynucleotide kinase (New England Biolabs, Beverly, MA), deionized water). DNA was purified by phenol-chloroform extraction and run on gel for quality control. The PCR product was added to a Ready-To-Go™ pUC18 Sma I/BAP + ligase kit (Amersham pharmacia biotech, Piscataway, NJ) containing a Sma I digest of pUC18. Ligations were set up according to manufacturers guidelines. Reactions were transformed in E. coli using the Epicurian Coli® XLII-Blue ultracompetent cells (Stratagen, La Jolla, CA) as described in 8.3.3. White colonies were screened further by colony hybridization, as described in 8.5, with a radiolabeled probe made from the HindIII - XbaI fragment of the M. sativa cDNA (nt270-1134 of GenBank L18899) purified from a 0.8% low melt point agarose gel in 1X TAE. A putative transformant was selected and its insert was sequenced with universal primers (Dr. Antony Leung, Fox Chase Cancer Center, Philadelphia, PA).
8.7.1.3 Subcloning in pPICZαC of the ENOD8 Expression Cassette

The pUC18 plasmid carrying the ENOD8 sequence was purified from the E. coli strain, which had been selected (8.7.1.2). The Xho I - Not I restriction digest of the plasmid (ENOD8 fragment and vector) was planned to be used for a ligation reaction together with a Xho I - Not I digest of pPICZαC. Both digests were purified by phenol-chloroform extraction. Ligation ratios were based on the mass of Xho I - Not I pPICZαC vector and on the mass of Xho I – Not I ENOD8 fragment by evaluation of DNA band intensity on an ethidium bromide – stained agarose gel. Ratios of vector mass versus insert mass were: 1:1, 3:1, 1:3, 6:1. Ligation was carried out as describe in 8.3.2, but for only 12 hours. 2 µl of ligation reactions were transformed in TOP10 competent cells (Invitrogen, Carlsbad, CA), according to the manufacturer instructions, which followed a 42°C heat-shock protocol. Transformants, which had recovered in SOC medium, were plated on LSLB (10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract) media supplemented with 10 µg/ml streptomycin, 25 µg/ml zeocin. The pPICZαC vector carries a gene conferring to E. coli resistance to the antibiotic zeocin in low salt conditions; this gene is not present on pUC18. Only the E. coli harboring a pPICZαC-based plasmid should grow. The colonies, which had grown on the transformation plates were re-streaked on LSLB media supplemented with 10 µg/ml streptomycin, 25 µg/ml zeocin. A few colonies were selected for plasmid extraction. The restriction pattern of one strain, which fitted the expected band size pattern was sent for sequencing (Dr. Antony Leung, Fox Chase Cancer Center, Philadelphia, PA). Primers recommended by Invitrogen were used for
sequencing: 5’AOX1 (5’ – GAC TGG TTC CAA TTG ACA AGC – 3’), 3’AOX1 (5’ – GCA AAT GGC ATT CTG ACA TCC – 3’), Alpha-factor (5’ – TAC TAT TGC CAG CAT TGC TGC – 3’).

8.7.1.4 pPICZαC-ENOD8 Transformation in *Pichia pastoris*

The pPICZα-ENOD8 construct was linearized with *Pme* I (New England Biolabs, Beverly, MA) within the 5’AOX1 region of the vector, which would result in its integration by gene insertion into the 5’AOX1 region of the *Pichia* genome. The GS115 mutant, growing only on histidine-supplemented media, and the X-33 wild-type were used for transformation. Transformation was carried out by heat-shock at 42°C of chemically competent *Pichia* prepared with the EasyComp™ transformation kit (Invitrogen, Carlsbad, CA), as described in the EasySelect™ *Pichia* expression kit (Invitrogen, Carlsbad, CA). Yeast transformants were selected by growth on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) supplemented with zeocin 100 µg/ml.

8.7.1.5 Mut Phenotype of *P. pastoris* Transformants

Transformants were tested for their methanol utilization phenotype (Mut+: fast, or Mut−: slow). All transformants were streaked on both MDH (1.34% YNB, 4.10⁻⁵ biotin, 2% dextrose, 0.004% histidine) and MMH (1.34% YNB, 4.10⁻⁵ biotin, 0.5% methanol) media, and grown for at least 2 days at 30°C. Transformants growing at the same rate on both media were Mut+. The Mut+ strains were used for expression studies.
8.7.1.6 Conditions for ENOD8 Expression in P. pastoris

Mut⁺ GS115 and Mut⁺ X-33 strains carrying the pPICZαC-ENOD8 construct were induced according to instructions from the EasySelect™ *Pichia* expression kit (Invitrogen, Carlsbad, CA). Briefly, a single *Pichia* colony was used to inoculate BMGH (100 mM potassium phosphate pH 6.0, 1.34% YNB, 4.10⁻⁵% biotin, 1% glycerol, 0.004% histidine) or BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4.10⁻⁵% biotin, 1% glycerol) in a flask. Growth was done at 30°C with 250-300 rpm shaking until OD₆₀₀ reached 2-6 (approximately 16-18 hours). The cells were pelleted to replace the growth media with BMMH (BMGH media whose 0.5% methanol is substituted for glycerol), or BMMY (BMGY media whose 0.5% methanol is substituted for glycerol). Expression was carried out in baffled flask covered with gauze to maximize aeration. Induced cultures or aliquots were centrifuged for further analysis by SDS-PAGE and western blot.

8.7.1.7 PCR Analysis of P. pastoris Putative Transformants

*Pichia* putative transformants, which had been selected by growth on zeocin-supplemented media (8.7.1.4) were directly tested by PCR for the presence of the ENOD8 expression cassette, with the 5’AOX1 and the 3’AOX1 primers recommended in the EasySelect™ *Pichia* expression kit (Invitrogen, La Jolla, CA), (Linder et al., 1996). Strains were grown in 3 ml of YPD (1% yeast extract, 2% peptone, 2% dextrose).
Yeast cells were pelleted and resuspended in deionized water to dilute them 10 times relative to their original density. 100 µl of the diluted yeast solution was mixed with 50 µl of 15 U/µl lyticase (#L2524, Sigma, St. Louis, MO) solution. Mixture was incubated for 20 min, with gentle re-mixing after the first 10 min of incubation. Each sample was then microwaved for 2 min, followed by immediate dipping in liquid nitrogen. Tubes were then left to thaw at room temperature. 5 µl of yeast extract was then used as template for PCR. Chemical concentrations were as follows in PCR tubes: 1X buffer, 2 mM MgSO₄, 2.5 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 5 µM of each primer, 0.25 U of Vent polymerase (New England Biolabs, Beverly, MA). Thermal file was: 2 min at 95°C, followed by 30 cycles with a denaturation step (1 min at 95°C), an annealing step (1 min at 54°C), an extension step (1 min at 72°C). PCR was completed with an 8 min incubation at 72°C to complete the extension of all PCR products.

8.7.2 Escherichia coli – Maltose Binding Protein Expression System

8.7.2.1 Construction of the Fusion Plasmid

The nt100 to nt1158 of the M. sativa cDNA GenBank L18899) was engineered by PCR. The ForpMAL primer (5’ – GCA CCC CGG GAC ACA TTG TGA TTT TCC TGC C – 3’) added a Sma I site at the 5’ end of the PCR product. The RevpMAL primer (5’ – GCA CGT CGA CTC ACT TTC TAT AAC ATG CCA TAT CTA G – 3’) added a Sal I site to the 3’ end of the PCR product. For both primers, the 4 nucleotides adjacent to the 5’ site of the Sma I and Sal I restriction site were added to increase the efficiency of digestions.
with these enzymes. A PCR reaction was setup as described earlier (8.7.1.1). The PCR product was run on a regular agarose gel. Localization of the piece of agarose to be cut out of the gel, was done under UV light. A neighbor lane with the PCR product band was exposed to UV light, but the sampled piece of agarose containing the DNA needed for further processing was never exposed to UV light. Purification of the PCR product from the agarose block was undertaken with the Sephaglas™ BandPrep kit (#27-9285-01, Amersham Biosciences, Piscataway, NJ), according to the manufacturer instructions. The PCR product was sequentially digested with \textit{Sma I}, then with \textit{Sal I}. Similarly the pMal-p2X vector was sequentially digested with \textit{Xmn I}, then with \textit{Sal I}. Both digests were purified by a phenol-chloroform extraction step. Aliquots of the vector and insert were run on agarose gel: after ethidium bromide staining, the concentration of DNA for both was evaluated. Ratios of vector mass versus insert mass were: 1:1, 1:2, 1:4. Ligation was set up as described in 8.7.1.3, but only for 4 hours. TOP10 competent cells (Invitrogen, La Jolla, CA) were transformed with 2 µl of ligation reaction, as described in 8.7.1.3. Recovered transformants were plated on LB with 10 µg/ml streptomycin and 100 µg/ml ampicillin, also supplemented with X-Gal and IPTG for blue/white selection as describe in 8.3.3. Plasmids were extracted from several white colonies. Strain #6 which showed the adequate restriction pattern was sent for sequencing (Lone Star Labs, Inc., Houston, TX). Primers used: malE (5’ – GGT CGT CAG ACT GTC GAT GAA GCC – 3’), M13/pUC (5’ – CGC CAG GGT TTT CCC AGT CAC GAC – 3’). 8.7.2.2 Conditions for \textit{ENOD8} Expression in \textit{E. coli}
Expression trials were set up according to guidelines from the manual of the pMAL™ protein fusion and purification system (New England Biolabs, Beverly, MA). Briefly, the MBP-ENOD8 expression strain and the strain with the vector without the ENOD8 sequence were used to inoculate LB media supplemented with 10 μg/ml streptomycin, and with 100 μg/ml ampicillin. Inoculated media was incubated at 37°C on a shaker, until $A_{600} \sim 0.5$. Before induction, a small volume of the media was aliquoted and its pellet kept at -80°C. IPTG was then added to the culture to a final concentration of 0.3 mM to induce recombinant expression. After induction completion, cells were collected by centrifugation.

8.7.2.3 Purification of the MBP-ENOD8 Fusion Protein

8.7.2.3.1 Periplasm Fraction Isolation by Cold Osmotic Shock

To purify protein from the periplasm, the induced bacterial pellet was resuspended in 30 mM Tris-HCl pH 8.0, 20% sucrose. 8 ml of solution was used per 0.1 of cells wet weight. EDTA pH 8.0 was added to the suspension to a final concentration of 1 mM. Mixture was incubated at room temperature for 10 min. Cells were pelleted by centrifugation at 8000 g at 4°C for 10 min. After discarding the supernatant, the pellet was resuspended in ice-cold 5 mM MgSO₄ to induce a cold osmotic shock to recover the periplasmic proteins. Mixture was left on ice-water for 10 min, with occasional shaking. After centrifugation, the supernatant was collected and used for further analysis.
8.7.2.3.2 Separation of Soluble and Insoluble Fractions

The induced bacterial pellet was resuspended in column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA pH 8.0), stored overnight at -80°C, then thawed on ice-water. Cells were lysed by sonicating them (Dr. O’Donovan lab, University of North Texas, Denton, TX) or by passing them through a French press (Dr. Kunz lab, University of North Texas, Denton, TX). Centrifugation was done to separate soluble and insoluble fractions used in further analysis.

8.7.2.3.3 MBP-ENOD8 Purification by Binding to Amylose Resin

Volumes used during purification were adjusted to precisely keep the proportions indicated in the manual of the pMAL™ protein fusion and purification system’ (New England Biolabs, Beverly, MA). Centrifugation of mixtures containing amylose resin was done at or below 6000 g. Eppendorf tubes containing 200 µl of amylose resin were spun briefly. After removal of the supernatant, the resin was resuspended in 1.5 ml of column buffer. After repeating this step once, the resin was resuspended in 200 µl of column buffer. Subsequently, 50 µl of crude extract was mixed with 50 µl of amylose resin slurry. Mixture was left to incubate on ice for 15 min, then centrifuged. The supernatant was discarded and the pellet washed four times with column buffer. The expected recombinant maltose binding protein–ENOD8 protein was eluted from the
amylose beads by incubating the slurry in column buffer with 10 mM maltose. After centrifugation, the supernatant was collected and used for further analysis.

8.7.2.4 MBP-ENOD8 Cleavage with Factor Xa

The MBP-ENOD8 fusion protein and the MBP-βGal control were incubated with factor Xa (New England Biolabs, Beverly, MA) in column buffer at room temperature for 24 hours. Each reaction contained 1 to 3.5 μg of factor Xa.

8.7.3 Nicotiana benthamiana Expression System

8.7.3.1 Plasmid Expression Construct

Constructs, based on pCambia vectors, were made by Dr. R. Dickstein.

8.7.3.2 Induction of ENOD8 Expression in Leaves of N. benthamiana

Leaves of N. benthamiana were infiltrated with induced culture of A. tumefaciens prepared by Dr. P. Neogi, according to published procedures (Voinnet et al., 2003). After infiltration, plants were placed back in a growth room (Dr. Chapman lab, University of North Texas, Denton, TX). At various times post-infiltration, recombinant expression levels were assessed. Leaves infiltrated with A. tumefaciens carrying a GFP construct were observed with a fluorescent stereomicroscope (Dr. Padilla lab, University of North Texas, Denton, TX). Leaves infiltrated with A. tumefaciens carrying the ENOD8 expression construct were analyzed by western blot.
8.8 Antisera

8.8.1 Anti-ENOD8 Oligopeptide Antisera

Dr. Dickstein and previous Dickstein lab members raised a polyclonal rabbit antisera against the oligopeptide CKNPSTRITWDGTHYTEAA conjugated to KLH by Biosynthesis, Inc. (Lewisville, TX) (Dickstein et al., 2002). The oligopeptide corresponds to amino acids 336-354 of the M. sativa ENOD8 putative polypeptide sequence (GenBank AAB41547). There is 89% identity between the M. truncatula’s predicted ENOD8 protein (GenBank AAC26810).

8.8.2 Affinity Purification of the Anti-ENOD8 Oligopeptide Antibody

8.8.2.1 Conjugation of the ENOD8 Peptide on a SulfoLink® Column

Purification was performed precisely according to the instructions from the SulfoLink® kit #44895 (Pierce, Rockford, IL). First, 1.5 mg of ENOD8 peptide, CKNPSTRITWDGTHYTEAA (SynPep, Dublin, CA) was resuspended in 2.5 ml of SulfoLink® coupling buffer (50 mM Tris, 5 mM EDTA-Na, pH 8.5). The A$_{280}$ was measured to confirm the concentration of peptide solution. The ENOD8 peptide absorbance at 280nm is due to its unique tryptophane residue and to its unique tyrosine residue. The resulting extinction coefficient of the ENOD8 peptide should be: \( \varepsilon = 5560 \text{ cm}^{-1} \cdot \text{M}^{-1} + 1200 \text{ cm}^{-1} \cdot \text{M}^{-1} \). The molecular weight of the ENOD8 peptide is 2151.3 g.mol$^{-1}$. The concentration of ENOD8 peptide in solution was calculated as follows: \( (A_{280} \times 2151.3)/(1 \times 5560 + 1 \times 1200) = \text{concentration in mg/ml} \). A SulfoLink® column was
equilibrated at room temperature. Through the entire procedure, the gel bed was never allowed to dry: there was always a small layer of buffer on top of it. The column was capped at both ends. The top cap was always removed first, then the bottom cap was removed to avoid drawing air into the gel bed. The storage buffer was drained from the column, which was then equilibrated with 8 ml of SulfoLink® coupling buffer. The 2.5 ml of ENOD8 peptide solution was then loaded on the column. After capping the column at both ends, it was left to mix on a meridian rotator (Labquake® shaker, Labindustries, Inc. – Berkeley, CA) for 15 min at room temperature. The column was then incubated at room temperature for an additional 30 min without mixing. The solution was drained from the column, which was then washed with 6 ml of SulfoLink® coupling buffer. The iodoacetyl groups, which had not been conjugated with the terminal cysteine of the ENOD8 peptide were blocked by incubation of the column with 2 ml of SulfoLink® coupling buffer containing 0.05 M of L-cysteine-HCl from the SulfoLink® Kit (Pierce, Rockford, IL). After capping the column at both ends, it was left to mix on a meridian rotator (Labquake® shaker, Labindustries, Inc. – Berkeley, CA) for 15 min at room temperature. The column was then incubated at room temperature for an additional 30 min without mixing. The column was then washed with 12 ml of SulfoLink® wash solution (Pierce, Rockford, IL). The column was subsequently washed with 4 ml of 1X PBS containing 0.05% NaN₃. After replacing the bottom cap, 2 ml of 1X PBS with 0.05% NaN₃ was added to the column. A porous disc was inserted in the column and left 1mm over the top of the gel bed. The disc prevents the gel bed from drying out by
automatically stopping the column flow when solution has drained down to the top of the gel bed. The ENOD8 peptide–conjugated SulfoLink® column was stored at 4°C.

8.8.2.2 Purification of ENOD8 Antibodies with the ENOD8 Peptide Column

0.5 ml of anti-ENOD8 oligopeptide antisera was spun for 30 min at 13,000 rpm at 4°C in the microcentrifuge. The supernatant was collected and mixed with 1 ml of 1X PBS. The ENOD8 peptide column was equilibrated at room temperature, then washed with 6 ml of 1X PBS. The 1.5 ml solution of anti-ENOD8 oligopeptide antisera was applied on the column and allowed to completely enter the gel bed. Then 0.2 ml of 1X PBS was applied to the column and allowed to enter the gel bed. Once the bottom cap was replaced on the column, 0.5 ml of 1X PBS was applied on the column. The top cap was replaced on the column, which was left to incubate at room temperature for one hour. The column was then washed with 12 ml of 1X PBS. The anti-ENOD8 oligopeptide antibody was eluted from the column by applying on it 8 ml of 0.2 M glycine pH 2.0. Eight fractions of 1 ml each were collected in Eppendorf tubes pre-filled with 50 μl of 1 M Tris pH 9.2. The A_{280} of the collected fractions was measured. Fraction number 3 had the highest absorbance (~0.32). Fraction number 4 and 5 contain some eluted antibodies (A_{280} = 0.13, A_{280} = 0.06, respectively). These three fractions were combined. The buffer in which the eluted antibodies were suspended was replaced by ultrafiltration, with an Ultrafree®-4 centrifugal filter unit spin column with a 50 kDa molecular weight cut off (Millipore, Bedford, MA). The pooled fraction were loaded on the spin column together with 1 ml of filter-sterilized 1X PBS. The spin column was
centrifuged at 4°C at 1,800 g, until the volume of retentate was approximately 100 µl. The spin column was then reloaded with 4 ml of filter sterilized 1X PBS. The column was centrifuged as before, until the volume of retentate was 300 µl. 3.7 ml of filter-sterilized 1X PBS was added to the spin column, which was centrifuged as before, until the volume of retentate was approximately 50 - 60 µl, as measured with a micropipettor. The affinity-purified anti-ENOD8 oligopeptide antibody was aliquoted in several tubes and stored at -80°C. A Bradford assay was carried out on the affinity-purified anti-ENOD8 oligopeptide antibody, which concentration was found to be approximately 3.6 µg/µl.

8.8.3 MBP-ENOD8 Polyclonal Antiseras

The MBP-ENOD8 recombinant protein was expressed and purified as described earlier (8.7.2.2, 8.7.2.3). Slight changes in the expression were used to try to maximize the amount of soluble MBP-ENOD8, which could be recovered. Induction was set at 15°C or room temperature, with IPTG concentration decreased to 0.03 mM. Induction was carried out for 12-16 hours induction with 280 rpm shaking. After purification the MBP-ENOD8 fusion protein concentration was evaluated by Bradford assay. The purity of the MBP-ENOD8 fraction was also checked by SDS-PAGE. A The purified MBP-ENOD8 protein fusion (110 µl, 30 µg/µl) was given to Bio-Synthesis (Lewisville, TX) to raise the antisera by immunizing two rabbits. The corresponding preimmune and polyclonal antisera were subsequently characterized by western blot.
8.9 Western Analysis

8.9.1 Protein Extraction from Plant Tissues

8.9.1.1 *M. truncatula* Roots

Soluble proteins were extracted as follows from root systems of ENOD8-RNAi *M. truncatula* transgenic plants. Root samples were taken out of -80°C storage and kept in liquid nitrogen in a Dewar container. The roots were grinded in an Eppendorf tube with a plastic pestle (Bel-Art Products). The pestle was dipped in liquid nitrogen before grinding to avoid any thawing of the root sample. During the grinding, the root material was kept over the liquid nitrogen of the Dewar container, and dip into it occasionally, to maintain it frozen. To maximize the efficiency of tissue grinding, the pestle was used in such a way to avoid crumpling all the root tissues down the bottom of the tube, from the start of the grinding process. Instead, the pestle was carefully handled past most of the root system down to the bottom of the tube. Small amount of tissues were crushed into a fine powder between the entire surface of the cone-shaped pestle and the walls of the tube. Subsequently, the pestle was lifted up the tube a little bit to grind some more tissue, using the same procedure. Once all the root tissue for one sample had been grinded, it was transferred on ice. Extraction buffer was subsequently added to the grinded roots, while they were on ice. This extraction buffer had been used previously in the extraction of *ENOD8* proteins from nodules of *M. sativa* (Pringle and Dickstein, 2004). A protease inhibitor cocktail was added to the extraction buffer. The composition of the buffer was as follows: 100 mM NaCl, 100mM Tris pH 7.4, 2X Halt™
protease inhibitor cocktail from the Halt™ protease inhibitor cocktail kit (Pierce, Rockford, IL), 1X EDTA solution from the Halt™ protease inhibitor cocktail kit (Pierce, Rockford, IL). Extraction buffer was added to tubes, while they were still on ice; volume added ranged from 50 µl for the largest amounts of tissues, to 20 µl for the smallest amounts of tissues. The extraction buffer and the tissue were homogenized by moving the pestle. All tubes were left on ice for 5 min, and then spun at 13,000 rpm in a microcentrifuge. The supernatants were transferred into fresh tubes. The protein concentration of each sample was evaluated by Bradford assay with 2 µl of the supernatant. Samples were then further analysed by SDS-PAGE and western analysis, as described in 8.9.2 and 8.9.3 respectively.

8.9.1.2 *N. benthamiana* Leaves

Leaves samples were taken out of -80°C storage. Subsequently, leaves were transferred in a porcelain mortar, which had been kept wrapped in foil at -80°C. Liquid nitrogen was added to the leaves, which were then grinded in the mortar, with a porcelain pestle. After the frozen powder was transferred to an Eppendorf tube containing 200 µl of extraction buffer (100 mM Tris pH 7.4, 100 mM NaCl, 3 mM EDTA). A protease inhibitor cocktail (#P9599, Sigma, St. Louis, MO) had been added to the buffer, according to the recommended usage from the manufacturer. The protease inhibitor cocktail contained protease inhibitors with broad specificity for the inhibition of serine, cysteine, aspartic, metalloprotease, and aminopeptidase. The supernatant was recovered by centrifuging the extract at maximum speed at 4°C in a microcentrifuge.
The supernatant was transferred to a fresh tube and analyzes by SDS-PAGE and Western analyses as described in 8.9.2 and 8.9.3 respectively.

8.9.2 SDS-PAGE Analysis

For the RNA interference analysis, 4-15% Tris-HCl precast gels were used (161-1104, Ready Gels, Bio-Rad, Hercules, CA). Otherwise SDS-PAGE gels were cast in the lab (Ausubel, 1987). First a 10% acrylamide solution (0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.05% APS, 0.05% TEMED) was poured in between two glass plates to cast the separating portion of the gel. The top of the casting acrylamide solution was covered with n-butanol. After the solution had solidified, the n-butanol was removed and the top of the separating portion of the gel thoroughly rinsed with deionized water. A 4% acrylamide solution (0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.05% APS, 0.1% TEMED) was poured on top of the separating gel for making the stacking portion of the gel. Very quickly, a comb was inserted in between the two glasses for making the wells. Cast gels were installed in an electrophoresis apparatus. Both inner and outer chambers were filled with 1X SDS-PAGE buffer (25 mM Tris, 0.19 M glycine, 3.5 mM SDS) or with 1X TGS (161-0772, Bio-Rad, Hercules, CA) when the Bio-Rad precast gels were used. After removing the comb, the wells were flushed with the electrophoresis buffer using a syringe to remove debris like small unpolymerized acrylamide, which could interfere with the protein entering the gel. Protein samples were mixed with 6X sample buffer (0.24 M Tris-HCl pH 6.8, 30% glycerol, 12% SDS, 0.6 M DTT, 12% bromophenol blue), for its concentration to be in 1X in the mixture. Samples were boiled for 2-3 min. After
they cooled down, samples were flash centrifuge, then pipeted in wells of the gel. A prestained ladder, precision plus protein™ dual color standards, 5 µl, (#161-0374, Bio-Rad, Hercules, CA) was loaded in one or more wells. Gels were run at voltage ranging from 75 V to 150 V at 4°C until the bromophenol blue front line ran out of the edge of the gel.

8.9.3 Protein Blotting on Nylon Membrane

After completion of the SDS-PAGE phase, gels were submerged in transfer solution (25 mM Tris base, 192 mM glycine, 10% methanol). A pair of 3MM Whatman filter paper and a nylon membrane were cut off to the dimension of the gel and equilibrated in transfer buffer. The nylon membrane was first wetted in 100% methanol, then rinsed in deionized water. On the cathode side of gel holder cassette, a protein sandwich was build by layering the following components in this sequential order: fiber pad, filter paper, gel, nylon membrane, Immobilon-P (Millipore, Bedford, MA), filter paper, fiber pad. Once closed, the gel holder cassette was holding very tightly the blotting sandwich, so that none of its components could slide relative to each other during protein transfer. Transfer was performed for a minimum of 16 hours at 4°C, with continuous stirring of the transfer buffer. Voltage was set at 70 V. After blotting was stopped, the nylon membrane was soaked briefly in 100% methanol, completely air-dried and wrapped in Saran wrap for storage at 4°C.
8.9.4 Coomassie Stain for Total Protein Detection

8.9.4.1 SDS-PAGE Gel Stain

All incubation steps were performed at room temperature. SDS-PAGE gels were incubated in staining solution for various length of time (50% methanol, 10% acetic acid, 0.05% Coomassie brilliant blue R250). Afterwards, stained gels were incubated in destaining solution (5% methanol, 7% acetic acid) until all areas of the gel devoid of protein were completely destained. Gels were dried as described in 8.10.3.

8.9.4.2 Nylon Membrane Stain and Destain

Blots were first wetted in 100% methanol. A solution of 0.1% Coomassie brilliant blue R in 50% methanol, 7% acetic acid was used to stain the blot for 2 min. Destaining of the blot proceeded first by incubation for 10 min in 50% methanol, 7% acetic acid. Signal to noise ration was further improved by incubating the blot in 90% methanol, 10% acetic acid for 10 min. The blots were completely dried before they were scanned with a calibrated densitometer GS-800 (Biorad, Hercules, CA). For further use in western analysis, the blots were completely destained in 100% methanol for a minimum of 6 hours, with solution change every hour.

8.9.5 Immunodetection with Alkaline Phosphatase

Protein blots were incubated in various solutions under constant gentle shaking on a horizontal shaker at room temperature. Blots were wetted in 100% methanol, then
incubated in deionized water for 2 min, and in 1X TBS (2.5 mM Tris-HCl pH 7.4, 10 mM NaCl), for at least 5 min. The blots were then covered with blocking buffer (1% BSA, 1% NGS, 0.02% NaN₃, 0.05% Tween 20, 1X TBS) and incubated for 16 hours. Blocking buffer was replaced by a primary antibody solution diluted in blocking buffer. Blots were incubated for one hour. Subsequently, a 5 min wash with 1X TBS was done; it was repeated for a total of five times. The blots were then incubated for one hour with a 1:2,000 dilution of a secondary antibody conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, inc. West Grove, PA). Subsequently, a 5 min wash with 1X TBS was done; it was repeated for a total of five times. The blots were equilibrated with 0.1 M Tris pH 9.6 during two incubations lasting at least 5 min each. For developing, the membranes were transferred in a chromogenic solution (9 ml of 0.1 M Tris-HCl pH 9.6, 100 µl of 5 mg/ml BCIP in DMF, 1 ml of NBT (prepared by dissolving 10 mg of NBT in 100 µl of DMSO, then adding the volume to 10 ml of 0.1 M Tris pH 9.6), 40 µl of 1 M MgCl₂). The developing reaction proceeded without agitation of the blot. Purple bands would appear in a few minutes. Once the signal to noise ratio was optimal, the reaction was stopped by covering the blot with TBST (1X TBS, 0.05% Tween 20). The blot was dried in between filter papers, then scanned with a calibrated densitometer GS-800 (Biorad, Hercules, CA) or a regular scanner.

8.9.6 Western Colony Blot for MBP-ENOD8 Detection

A ‘no-induction’ plate was made with LB media supplemented with 10 µg/ml streptomycin and 100 µg/ml ampillicin. The media of the ‘induction’ plate was
additionally supplemented with 80 μg/ml X-Gal, 0.3 mM IPTG. A nylon membrane, Immobilon-P (Millipore, Bedford, MA) was laid on top of the media of two ‘no-induction’ plates. MBP-ENOD8 E. coli transformants were streaked on both plates. After colonies had appeared on both plates, one membrane was transferred on top of the media of an ‘induction’ plate. Induction lasted for 7.5 hours. The two membranes were then processed for immunodetection. They were laid on top of a stack of filter paper soaked in 10% SDS for 10 min. The membranes were then washed 4 times by laying them on top of 1X TBS soaked filter paper. Membranes were then dipped in 100% methanol for 15 sec, then air-dried. Immunodetection was subsequently carried out as described earlier 8.9.4.

8.10 Esterase and Lipase Assays

8.10.1 Lipolytic Activity Assay on Bacterial Colonies

8.10.1.1 Tween Plate Assays

Bacterial growth media contained: 10 g/l bacto-peptone, 5 g/l NaCl, 0.1 g/l CaCl₂·H₂O (Sierra, 1957). Media was supplemented with 0.1% Tween 20 or 0.1% Tween 80, and with antibiotics (10 μg/ml streptomycin, 50 μg/ml ampicillin) and with 0.3 mM IPTG when appropriate. E. coli strains expressing the MBP-ENOD8 fusion protein, and Pseudomonas aeruginosa (Dr. O’Donovan’s lab, University of North Texas, Denton, TX) were streaked on the Petri dishes. Plates were incubated at 37°C. Plates were inspected for the formation calcium salt crystals with a stereomicroscope.
8.10.1.2 α-Naphthylacetate Filter Paper Assay

LB media (Sambrook et al., 1989) supplemented with 10 μg/ml streptomycin, 50 μg/ml ampicillin, 0.3 mM IPTG was used. A filter paper was laid flat on the media and streaked with the MBP-ENOD8 recombinant E. coli or the E. coli strain carrying the expression vector without the ENOD8 sequence. Plates were incubated at 37°C for bacterial growth and induction of recombinant expression. The filter paper carrying the bacterial growths was transferred in 0.1 M sodium phosphate pH 7.4. α-naphthylacetate was added to a concentration of 0.6 mg/ml. After the α-naphthylacetate had dispersed in the solution, Fast Blue B (#44660, Fluka, Milwaukee, WI) was added to the solution to a concentration of 1 mg/ml. One would expect to see purple staining appear around colonies with hydrolytic activity. Incubating the filter paper in 20% methanol, 10% acetic acid solution for 15 min, stopped the colorimetric reaction.

8.10.2 Spectrophotometric Assay with Purified MBP-ENOD8 Protein

Esterase activity was measured by monitoring the rate of hydrolysis of p-nitrophenylacetate in p-nitrophenol at 410nm or by monitoring the rate of α-naphthylacetate in α-naphthol at 321nm. Purified MBP-ENOD8 fusion protein, factor Xa digests of the MBP-ENOD8 fusion protein, a porcine esterase positive control (E-3019, Sigma, St. Louis, MO) were tested. The mass of MBP-ENOD8 was evaluated by doing a Bradford assay (Ausubel, 1987). 1 to 10 μg of MBP-ENOD8 protein were added to the assay mixture (0.02 M HEPES pH 7.4, 0.1 M NaCl, 1% Na deoxycholate, 3%
acetonitrile, 1 mM of p-nitrophenylacetate or α-naphthylacetate dissolved in acetonitrile). Absorbance change was measured as soon as MBP-ENOD8 was mixed in the assay cuvette.

8.10.3 SDS-PAGE Esterase Assay

Protein samples were run on a regular protein polyacrylamide gel by SDS-PAGE. After completion of the electrophoresis, the gel was incubated in a wash solution (0.1 M sodium phosphate pH 7.4, 0.5 mg/ml BSA, 0.1% Triton X-100) to remove SDS. Three one-hour incubations were done at room temperature. The gel was then transferred in a 6 M urea solution to denature the proteins. For protein renaturation the gel was then incubated for 15 min in solution with decreasing urea concentrations: 3 M, 1.5 M, 0.75 M, 0.38 M, 0.19 M, 0.09 M, 0.05 M. The gel was then transferred to a 0.1 M sodium phosphate pH 7.4 solution. For the esterase assay, the gel was transferred to a container filled with 200 ml of 0.1 M sodium phosphate pH 7.4. Subsequently, 2 ml of 60 mg/ml α-naphthylacetate in acetone were added to the gel in the container. 200 mg of Fast Blue B (#44660, Fluka, Milwaukee, WI) in 10 ml of deionized water were added to the gel in the container. Protein bands with esterase activity would stain in purple. Staining reaction was stopped by transferring the gel in a 20% methanol, 10% acetic acid solution. For the purpose of keeping a permanent record of the experiment, stained gels were scanned or dried between two gel drying films. Before gels were dried, they were extensively rinsed in deionized water for removing any trace of organic solvent and acetic acid.
8.11 Histochemical Staining of *M. truncatula* Nodules

All *ENOD8* promoter GUS *M. truncatula* plants tested were T₁ progenies from T₀ plants that had been regenerated from callus #10 by Dr. Purnima Neogi.

8.11.1 GUS Activity Detection

Nodules at various developmental stages were collected from nodulated roots of *M. truncatula ENOD8* promoter GUS plants. Nodules were not fixed. Mature nodules (15 dpi old or older) were hand sectioned with a single edge razor blade (No. 9). Young nodules (less than 15 dpi) were not sectioned. Immediately after sampling, nodules were dipped in a GUS staining solution (1 mM X-gluc, 0.1% Triton, 1X phosphate buffer, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide). Nodules from *ENOD11* promoter GUS transgenic *M. truncatula* (Journet et al., 2001) were used as a positive control, to verify that the GUS stain solution was working. GUS stain solution was vacuum-infiltrated in samples 4 times for 30 sec. Nodules were then incubated at 37°C. The extent of the GUS stain was checked periodically with the naked eye or with a stereomicroscope. Within a few hours, *ENOD8* promoter GUS stain was visible. Samples were not left to overstain, in order to avoid diffusion of GUS stain to areas of the nodules where there was actually no GUS expression. GUS stained nodules were put at 4°C. When nodules were left at 4°C in GUS stain solution, no increase of blue staining was observed thereafter. Alternatively, nodules were transferred in a tube containing 1X phosphate buffer; storage did not result in detectable loss of GUS stain. Some GUS stain nodules were post-stained with iodine, as described below.
8.11.2 Iodine Staining

Nodule sections were incubated in iodine solution (5% iodine, 10% potassium iodide, 10% acetic acid) to detect the accumulation of starch granule which is a marker of the interzone II-III (Vasse et al., 1990). Sections were stained for 20-30 sec. Excess staining was removed by incubating sections in 1X phosphate buffer for up to 24 hours.

All nodule sections were subsequently mounted in a drop of water on a glass slide and topped with a coverslip. Observations were made with an Olympus BX 50 microscope.

8.12 Immunolocalization in *M. truncatula* Nodules

8.12.1 Nodule Section Preparation

8.12.1.1 Nodule Sampling

Mature pink nodules were collected from *M. truncatula* A17 plants grown in aeroponics chambers. Nodulated roots were cut off the rest of the plants with a scalpel or a pair of scissors and immediately put in a Petri dish filled with deionized water. Segments of roots carrying single nodules were trimmed away from the rest of the root system, as follows. Under a stereomicroscope, nodules were separated from the root with a scalpel; tweezers were holding the root during sectioning. Nodules were picked from the deionized water-filled Petri dish a thin paintbrush and gently transferred on the tip of a pair of tweezer, from which they were dipped into a fixing solution.
8.12.1.2 Fixation of Nodule Tissues

8.12.1.2.1 Formaldehyde-Based Fixative

1 to 3% formaldehyde solutions were made in 1X phosphate buffer pH 7.0. 16% paraformaldehyde in distilled water stock solutions (Electron Microscopy Sciences, Hatfield, PA) were brought to the target concentration by addition of deionized water and 10X phosphate buffer (1.37 M NaCl, 27 mM KCl, 43 mM Na₂HPO₄, 14 mM KH₂PO₄). Fixative solution was infiltrated in the nodules, by applying vacuum 4-5 times for 30 sec to 1 min. After infiltration, nodules were left to at 4°C for 30 min to 15-17 hours.

8.12.1.2.2 Organic Solvent Fixation

96% ethanol, 100% methanol, and 100% acetone stocks were chilled at 4°C. Solvents were aliquoted in glass vials kept on ice until nodules were dipped in. Nodules would immediately sink to the bottom of the vials. Subsequently, vials were transferred at 4°C on a meridian rotator (Labquake® shaker, Labindustries, Inc. – Berkeley, CA), at low speed, for overnight incubation. For the cellular localization experiment, the methanol fixation step was preceded by light fixation with 0.5% formaldehyde in 1X phosphate buffer pH 7.0. Five to 7 vacuum infiltration rounds of 30 sec each were applied to the nodules. Then the nodules were left to incubate at room temperature for 20 min. Nodules were rinsed 6 times for 10 min in 1X phosphate buffer prior to fixing them with methanol.
8.12.1.3 Rinse of Fixed Nodules in Phosphate Buffer

After they were fixed, nodules were extensively rinsed in 1X phosphate buffer, in Petri dishes, at least 6 times for 10 min. Nodules, which had been incubated in organic solvents, tended to float on top of the buffer surface. With a small paintbrush, I gently pushed down the nodules until they were staying at the bottom of the Petri dish. Nodules were then kept in a Petri dish, in 1X phosphate buffer at 4°C for a few minutes before embedding.

8.12.1.4 Embedding of Nodules in Agarose

Fixed nodules were embedded in 6% low melt point agarose in 1X phosphate buffer. The low melt point agarose solution was boiled over a Bunsen burner until it was completely liquefied. Agarose was cooled down until the beaker containing it felt not too hot to the touch; this would also be useful to let the bubbles, which had formed during the boiling phase float up the surface of the agarose. A hot plate was kept nearby to re-warm the agarose as needed, to avoid premature gelling. Prior to embedding, nodules were examined with a stereomicroscope (Accuscope) to determine, which nodules had the best morphology and had not sustained damage during their sectioning from the root. For embedding, a regular glass slide was first placed on the stage of a stereomicroscope. A nodule was put on the tip of a thin paintbrush. With a Pipetman (P1000, Gilson – France), less than 400 µl of melted agarose were pipeted onto the slide. Immediately, the nodule was deposited on top of the melted agarose. More agarose was added on the top of the nodule to cover it completely. With a pair of
tweezers, the nodule was oriented in the agarose drop, for its longitudinal axis to be parallel to the surface of the slide. Several nodules were put on the same slide. Once a slide was full with embedded nodule, it was put at 4°C, inside a humidity chamber (large Petri dish lined with moist paper towel, on which there were two pieces of skewers running parallel to support the glass slides).

8.12.1.5 Sectioning of Nodule with a Vibratome

Sectioning was carried with a Vibratome® 1000 classic, (Vibratome, St. Louis, MO), (Dr. Fuchs’ and Dr. Schwark labs, University of North Texas, Denton, TX). Before sectioning, nodules were kept in humidity chambers on ice. With a single edge razor blade (No. 9), an agarose block with a nodule in it was trimmed into the shape of a cube. The long axis of the nodule should be confounded with an imaginary line joining the two centers of two parallel faces of the cube. The vibratome bath was filled with 1X phosphate buffer. A single edge razor blade, which had been cut in half, along its edge axis (biology building, machine shop), was installed in the blade holder, at an angle between 30° and 35° as measured with the blade angle indicator. The agar cube was affixed on a chuck with superglue in a way that the longitudinal axis of the nodule would be horizontal and perpendicular to the cutting edge of the blade. The amplitude of lateral vibration (perpendicular to the cutting edge of the blade) was set at 9.5, close to the maximum, which is recommended for soft tissues (Ruzin, 1999). The speed of advancement of the blade was set to the absolute possible minimum, which also recommended when sectioning soft tissues. The chuck with agarose block was installed
on the vibratome on a vise-type specimen holder. By turning a dial, the holder’s height was adjusted relative to the surface of the liquid in the vibratome bath, so that the agarose block was completely submerged. Enough buffer was added in the bath for the blade edge to be under the surface of the liquid. After shaving layers of agarose above the nodule with the vibratome blade, the dial was turned 180° clockwise to raise up the chuck holder by only 50 μm at a time in order to obtain nodule section of that thickness. A section floating in the vibratome bath would be immediately picked up with a thin paintbrush to transfer it in 1X phosphate buffer on a hanging-drop slide. A stereomicroscope was used to screen for sections, which were the most intact and which also be likely to include nodule zones I, II, III. Sections were scored and kept in 1X phosphate buffer in a 96-well tray. The blade would need to be replaced after one or two nodules had been completely sectioned.

8.12.1.6 Affixing Nodule Sections onto Glass Slides

3” by 1” x 1mm slides (Chase Scientific Glass) were spotted in the middle with 5-10 μl of 0.1% of poly-L-lysine, #P8920 (Sigma, St. Louis, MO). Slides were then placed on top of a hot plate to evaporate water. A small translucent disk in the middle of the slide indicated the presence of the dried poly-L-lysine. Nodule sections selected for immunostaining were transferred in 1X phosphate buffer on a hanging drop slide. In most cases, the agarose frame was still around the nodule sections. Under the stereomicroscope, the agarose was taken away from the nodule section, by gently twisting it with thin paintbrushes and/or with plastic tip thinned with a razor blade. Care
was taken not to brush the surface of the section at any time to avoid scratching away cells. A small drop of 1X phosphate buffer was placed on the area of the slide with the dried poly-L-lysine. The nodule section was carried with a thin paintbrush on top of the buffer above the poly-L-lysine. Under the stereomicroscope the section was oriented in a direction, which would facilitate microscopic observation later. To affix strongly the section on the surface of the glass slide, the section was completely dried. Excess liquid was first removed with a pipet, then Kimwipes were used to speed removal of liquid on the edge of the section. Care was taken to not touch the section, which would in deformation of the tissue. I checked with the stereomicroscope that the section was completely dried. Then I surrounded it completely by applying the tip of pen containing a hydrophobic medium, PAP pen (Research Products International Corp., Mt. Prospect, IL) creating a thin water-repellent film (PAP square). Once the film had dried, 1X phosphate buffer was added several times in the PAP square to verify that the section was firmly attached to the slide. Solutions were always pipeted in the corner of the PAP square, never directly on top of a section. If a section was getting even partially unstuck from the surface of the slide, it would have to be dried again in order to be reaffixed on the slide. Sections were completely covered with a 50 μl volume. Solutions were not pipeted in excess in the square to avoid an overflow of liquid over the edges. Loss of liquid could result in uncovering the section, which could become partially dried. All sections were kept wet during all the incubation steps of the subsequent immunostaining procedure.
8.12.2 Immunostaining of Nodule Sections

8.12.2.1 Incubations with Antibodies

    All incubations of nodule sections were done on slides within a PAP square, filled
with the appropriate solutions. During the incubations, slides were laying flat on moist
paper towels, and were covered with an upside down container, to keep the small
incubation volumes from evaporating. When changing solutions, the slides were tilted
to make the liquid fall from the slide onto the paper towel. Excess liquid remaining in
the PAP square was removed by applying a Kimwipe tissue on the slide. As much liquid
as possible was removed, without letting the sections dried. As soon as all the liquid
was removed form around a section, the solution for the next incubation step or wash,
was applied in the PAP square. First the sections were incubated for one hour at room
temperature with blocking buffer (in 1X PBS: 3% BSA, 5% NGS, 0.1% Tween20).
Sections were then incubated for one hour at room temperature with a solution
containing one or two primary antibodies. Each section was then washed twice in
blocking buffer. Sections were subsequently incubated for one hour at room
temperature with a solution containing one or two secondary antibodies. Each section
was then washed twice in blocking buffer.

8.12.2.2 Preparation of Primary Antibodies and Primary Antisera

    Antisera and affinity-purified antibodies were diluted in blocking buffer. The MBP-
ENOD8 antisera, and its corresponding preimmune sera were diluted 1:200 for alkaline
phosphatase detection and 1:500 for fluorescence microscopy. The anti-$ENOD8$
oligopeptide antisera, and its corresponding preimmune sera were diluted 1:500. The
histone-H1 antibody, clone AE-4, mouse monoclonal IgG$_{2a}$, (Upstate, Lake Placid, NY)
and the affinity-purified $ENOD8$ antibody were diluted 1:100. For the immunodepletion
experiment, the 1:100 affinity-purified $ENOD8$ antibody was pre-incubated with 5 µg or
50 µg of the $ENOD8$ peptide, CKNPSTRITWDGTHYTEAA (SynPep, Dublin, CA). As a
control a 1:100 dilution of the affinity-purified $ENOD8$ antibody was also pre-incubated
without the $ENOD8$ peptide, to verify that longer incubation times did not affect the
antibody binding ability for its target antigen.

8.12.2.3 Secondary Antibodies Dilutions

All secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West
Grove, PA) were diluted in blocking buffer. For alkaline phosphatase detection, I used a
1:200 dilution of an affinity-purified goat anti-rabbit IgG (H+L) conjugated with alkaline
phosphatase, #111-055-003 (Jackson ImmunoResearch Laboratories, inc, West Grove,
PA). For fluorescence microscopy various combinations of secondaries were used, their
JIR catalog number is in parentheses: FITC-conjugated goat anti-mouse IgM (H+L)
(#115-095-075), TRITC-conjugated goat anti-rabbit IgG (H+L) (#111-025-144), FITC-
conjugated goat anti-rabbit (H+L) (#111-095-144), TRITC-conjugated goat anti-mouse
(H+L) (#115-025-166), Cy5-conjugated goat anti-rabbit (H+L) (#111-175-144). All
fluorophore-conjugated antibodies were diluted 1:500 in blocking buffer. When
incubating a section with a secondary antibody, the corresponding humidity chamber
was covered with foil to avoid excess exposure to light, which could bleach the fluorophore prior to observations.

8.12.2.4 Alkaline Phosphatase Detection

After incubation with the secondary solution, sections were washed twice for 15 min in buffer (0.1 M Tris, 0.15 M NaCl, pH 9.5). A solution including 0.005% BCIP, 0.01% NBT, 5 mM magnesium chloride, and 0.1M Tris pH 9.5 was used to stain the samples. After about 25 min of incubation, excess solution was removed. Cover slips were applied on top of the sections. Observations were made with an Olympus BX50 microscope.

8.12.2.5 Nodule Sections Counterstaining with SYTO® 13 and Slide Mounting

After the washes following the secondary incubation, some sections were additionally incubated for 15 min at room temperature in 5 μM SYTO® 13, S-7575 (Molecular probes, Oregon, USA) in blocking buffer. Subsequently, sections were washed twice for 5 min in blocking buffer. For immunofluorescence observations, section were mounted with 3 μl anti-photobleaching media, ProLong® Gold antifade reagent, P36930 (Molecular probes, Oregon, USA). A coverslip was then laid on top of the sections. Sections were laid flat in the dark at room temperature overnight for the mounting media to cure the section. Coverslips were sealed by their edges onto the glass slide by application of nail polish.
Epifluorescence microscopy was done with a Zeiss Axioskop2 mot plus (Dr. Padilla’s lab, University of North Texas, Denton, TX). Images acquisition and processing was done with the Openlab software (Improvision, Lexington, MA). Confocal observations were done with a Perkin Elmer Ultraview ERS spinning disk confocal system (PerkinElmer life and analytical sciences, Inc., Boston, MA) connected to a Zeiss Axiovert 200M (Zeiss, Germany), with a 488nm (SYTO® 13), 568nm (TRITC), and a 640nm (Cy™5) laser lines. Observations were made at 20X and 100X (Dr. Kate Luby-Phelps, UT Southwestern, live cell core imaging facility, Dallas, TX). Images were acquired as z-stack using the software developed by PerkinElmer provided with the Ultraview ERS. Data was then processed with ImageJ from NIH (rsb.info.nih.gov/ij/).
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