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STRUCTURE, FUNCTION, AND REGULATION OF THE EARLY NODULIN GENE *ENOD2* FROM LEGUME PLANTS

By

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ABSTRACT

STRUCTURE, FUNCTION, AND REGULATION OF THE EARLY NODULIN GENE *ENOD2* FROM LEGUME PLANTS

By

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The early nodulin *SrEnod2* gene of the tropical legume *Sesbania rostrata* encodes a putative hydroxyproline-rich cell wall protein, and is expressed exclusively in the parenchyma cells of both stem- and root-nodules. In addition, the *SrEnod2* gene can be specifically induced in roots by the plant hormone cytokinin, and this induction occurs at a post-transcriptional level. One focus of this dissertation research is to elucidate the molecular mechanism that controls the nodule parenchyma-specific expression of the *SrEnod2* gene using both molecular genetic and biochemical approaches.

By analyzing chimeric reporter gene expression in transgenic *Lotus corniculatus* plants, a detailed characterization of the 5' and 3' *SrEnod2* flanking sequences has been carried out. These analyses indicate that the *SrEnod2* 3' untranslated region is both required and sufficient for gene expression in nodule parenchyma cells. Another finding is that the 5' flanking sequence does not appear to contain any significant *cis*-acting elements for nodule parenchyma-specific expression. This observation is in contrast with the report that the 5' upstream region of the soybean *Enod2*

gene directs nodule parenchyma-specific expression, indicating different mechanisms may be involved in regulation of the expression of these two genes.

Using an in-vitro protein-RNA UV-cross linking approach, several nodule-specific proteins have been identified to interact with the RNA transcribed from the *SrEnod2* 3' untranalated region. Competition experiments using both non-specific and specific competitors suggest that the *cis*-acting elements responsible for nodule parenchyma-specific expression co-localize with specific protein binding sequences.

Cytokinin up-regulation of the *Enod2* gene expression is not a common phenomenon among legume plants. To facilitate molecular and genetic analyses of the cytokinin regulation of the *Enod2* gene expression, the *Enod2* homologue was isolated from the model legume, *L. japonicus*. Northern blot analyses indicate that the *LjEnod2* gene is inducible in roots, but not by cytokinin. Submergence of the *L. japonicus* roots in water for several hours was sufficient to induce the *LjEnod2* gene expression. Further analyses demonstrate that ethylene is responsible for the *LjEnod2* gene expression in roots of *L. japonicus*.

To Yihong, June, Rong and my parents

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CHAPTER 1

INTRODUCTION

SECTION I. Symbiotic Nitrogen Fixation and Nodule Development

A. Symbiotic Nitrogen Fixation

Symbiotic nitrogen fixation takes place in the central infected cells of nodules, new plant organs formed on roots of legume and several non-legume plants after infection by soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. The bacterial enzyme nitrogenase catalyzes the following reaction:

$$N_2 + 8H^+ + 8e^- + 16\text{Mg-ATP} \rightarrow 2NH_3 + H_2 + 16\text{Mg-ADP} + 16\text{Pi} \quad (1)$$

In this reaction, atmospheric dinitrogen is reduced to ammonium, which is exported from the infected cells to adjacent non-infected cells and assimilated as a nitrogen-source for the plant host. Plants, in turn, supply the nitrogen-fixing bacteria with carbon sources to provide energy for the nitrogenase-catalyzed reaction. Nitrogenase consists of two components, the homodimeric Fe protein, encoded by the bacterial *nifH* gene, and the tetrameric molybdenum-iron (MoFe) protein, encoded by the *nifD* and *nifK* genes (Dixon et al., 1977; Ruvkun, et al., 1982). Since the MoFe protein is irreversibly denatured by oxygen (Appleby, 1984), nitrogen fixation requires low oxygen concentration in the infected cells of the nodule. On

the other hand, a large amount of energy (ATP) required for the nitrogen-reducing reaction has to be provided via oxidative processes, since rhizobia are generally aerobic bacteria. Thus, there is a high demand for oxygen influx into nodules. Legume plants have developed different strategies to cope with this "oxygen paradox". A low oxygen concentration in the nodule central nitrogen-fixing tissue is achieved by a combination of high metabolic activities of the bacteroids and the formation of an oxygen diffusion barrier in the periphery of the nodule (nodule parenchyma; Tjepkema and Yocum, 1974; Witty, 1986). In addition, and possibly most importantly, the oxygen carrier protein leghemoglobin provides oxygen buffering activity by transporting oxygen to the actively nitrogen-fixing and respiring bacteroids in the infected cells at a low intracellular free oxygen concentration (< 10 nM; Appleby, 1984).

B. Nodule Development

Nodule formation initiates with the exchange and recognition of signal molecules between the compatible symbiotic partners (Long et al., 1989; Fisher and Long, 1992; Figure 1-1). Flavonoids and isoflavonoids are signal molecules secreted by the roots of legume plants that serve as chemoattractants as well as inducers of bacterial nodulation (*nod*) genes (Peters et al., 1986; for a review, see Long, 1989). Expression of the *nod*

genes, in turn, results in the synthesis of lipochitooligosaccharides, known as "Nod factors", which can induce root hair deformation, cortical cell division and nodule formation at extremely low concentrations (10^{-8}-10^{-11} M; Lerouge et al., 1990; Truchet et al., 1991; Ardourel et al., 1994; Heidstra et al., 1994; Yang et al., 1994). The structure of Nod factors was first determined in *R. meliloti*, the microsymbiont of alfalfa (NodRm-1; Lerouge et al., 1990). NodRm-1 consists of a backbone of three to five β-1,4-linked *N*-acetylglucosamines bearing a C16 unsaturated fatty acid chain on the non-reducing sugar residue. The structure of Nod factors from many other rhizobia has subsequently been determined (for reviews, see Vijn et al., 1993; Denarie and Cullimore, 1993; Carlson et al., 1995). Now, it is clear that Nod factors generally have a N-acylated chitin oligosaccharide backbone and can have various substitutions on both the reducing and the non-reducing sugar residues. These substitutions likely render Nod factors specific for their host (Vijn et al., 1993; Denarie and Cullimore, 1993; Carlson et al., 1995).

Upon *Rhizobium* infection, root hairs undergo curling and deformation, and form characteristic structures called Shepherd crooks (Kijne, 1992). Bacteria are entrapped in these structures. A local hydrolysis of the plant cell wall and a subsequent plasma membrane invagination and deposition of new plant cell wall material result in the formation of a tubular structure, the so-called infection thread, by which the bacteria enter the plant (for reviews,

see Bauer, 1981; Newcomb, 1981; Brewin, 1991; Kijne, 1992). As the infection threads form, root cortical cells are mitotically reactivated to form the nodule primordium. Infection threads grow toward this primordium and release bacteria into the cytoplasm of nodule primordium cells in a process called endocytosis (Verma, 1992). Concomitant with the infection process, a meristem is formed at the distal part of the primordium and the meristem produces cells that differentiate into the nodule tissue (Nap and Bisseling, 1990; Kijne, 1992).

The site of first cortical cell divisions depends on the type of nodule formed upon rhizobial infection. In general, temperate legumes, including pea, alfalfa, clover, form indeterminate nodules, which have a persistent meristem at the apex. As a result of continuous cell divisions, new cells are added to the cortical and central tissues of emerging nodules. Consequently, all tissues of these nodules are of a graded age from the apical meristem to the root attachment point. The first cell divisions in roots of plants forming indeterminate nodules occur in the inner cortical cells (Nap and Bisseling, 1990). The infection threads must traverse the outer cortex before they reach these cells. Before infection thread penetration, the outer cortical cells undergo morphological changes. The nuclei move to the center of the cells, and the microtubules and the cytoplasm rearrange to form radially oriented structures, the cytoplasmic bridges, or pre-infection threads (van Brussel et

al; 1992). The infection threads traverse the outer cortical cells through these radially aligned cytoplasmic bridges.

In contrast, tropical legumes, such as soybean, *Lotus*, and *Sesbania*, form determinate nodules, whose meristems cease to divide shortly after rhizobial infection (Newcomb, 1981). As a result, development of determinate nodules largely depends on cell elongation and expansion. Within a determinate nodule, cells are at approximately the same developmental stage. In plants forming determinate nodules, first cell divisions occur in the root outer cortex. Subsequently, cell divisions also take place in the pericycle and inner cortex, several cell layers separated from the initial cell divisions. Eventually, the two meristematically active regions merge and give rise to the nodule tissues (Kijne, 1992).

C. Gene Expression During Nodule Development

During nodule development, a number of plant genes are induced or their expression is differentially enhanced. They are termed nodulin genes (Legocki and Verma, 1980; van Kammen, 1984; Verma and Delauney, 1988). Genes expressed during early stages of nodule development, well before the onset of symbiotic nitrogen fixation, are collectively called *e*arly *nod*ulin genes (*Enod*s). Those expressed around the onset of nitrogen fixation are termed late nodulin genes. Although the function of many

nodulin genes identified to date remains to be determined, the sequential expression of particular nodulin genes marks the different phases of nodule development (Scheres et al., 1990b; for reviews, see Nap and Bisseling, 1990; Sanchez et al., 1991; Mylona et al., 1995). Additionally, the spatial expression of nodulin genes in nodules clearly suggests their involvement in particular morphological and biochemical aspects of nodule development.

Nodulins involved in initial root hair deformation upon rhizobial infection, include RH-42 and RH-44, which were identified by *in vitro* translation of mRNAs isolated from root hairs (Gloudemans et al., 1989); and a lipid transfer-like protein (LTP), which was isolated from a cDNA library constructed from mRNAs of root hairs infected with rhizobia (Krause et al., 1994). The RH-42 gene is not expressed in root hairs of uninoculated pea plants, but induced in root hairs after infection with *R. leguminosarum* bv. *viciae* (Gloudemans et al., 1989). It is possible that the RH-42 is involved in root hair curing. The expression of the pea gene RH-44 is markedly stimulated in pea root hairs upon rhizobial infection, although this gene is also expressed at a low level in root hairs of uninoculated plants (Gloudemans et al., 1989). The mRNA encoding the LTP is expressed in all tissues, except nodules. However, the transcript level is induced in root hairs 24 hr after application of Nod factors, different plant hormones, or rhizobia (Krause et al., 1994).

Two early nodulin genes of pea (*Enod5* and *Enod12*) have been identified that are involved in the infection process (Scheres et al., 1990a). The expression of the *Enod12* gene can be detected in root hairs, root cortical cells and nodule cells containing growing infection threads (Scheres et al., 1990a). Interestingly, this gene is expressed not only in cells containing growing infection threads, but also in several cell layers located in front of the infection thread tips, where the cells are preparing for infection thread penetration (Scheres et al., 1990a). The spatial distribution of the *Enod5* mRNA in infected pea roots is strikingly different from that of the *Enod12* transcript. The *Enod5* gene is expressed only in cells containing the growing infection thread (Scheres et al., 1990b). In indeterminate pea nodules, the *Enod12* transcript is also detectable in cells adjacent to the distal meristem in the invasion zone, while the *Enod5* mRNA reaches a maximum level in the early symbiotic zone (Scheres et al., 1990b).

Several nodulin genes are expressed during mitotic reactivation of root cortical cells by rhizobia. They include *Enod12* (Scheres et al., 1990a), Gm93 (Kouchi and Hata, 1993), *Enod40* (Kouchi and Hata, 1993; Yang et al., 1993; Asad et al., 1994; Crespi et al., 1994; Matvienko et al., 1994), and MtPRP4 (Wilson et al., 1994). These genes are expressed in all cells of the developing nodule primordia. *Enod40* gene is particularly interesting, because it is also expressed at an early stage preceding the first cortical cell division in the region of the pericycle opposite to the protoxylem poles

(Kouchi and Hata, 1993; Yang et al., 1993; Asad et al., 1994; Matvienko et al., 1994).The *Enod40* gene has been shown to be inducible by Nod factors (Vijn et al., 1993; Yang et al., 1993). Expression of antisense *Enod40* gene in alfalfa arrests callus growth, while over-expression of the *Enod40* gene in alfalfa causes the formation of teratomas, a callus containing developing shoots and short roots (Crespi et al., 1994). Since the *Enod40* genes have no significant open reading frame, and *in vitro* translation of the *Enod40* transcripts does not yield any protein product, several groups of investigators have proposed that the *Enod40* genes play a role in plant development by acting as RNA (riboregulators; Asad et al., 1994; Crespi et al., 1994; Matvienko et al., 1994). Recently, van de Sande et al. (1996) have reported evidence to argue that an oligopeptide of about 10 amino acids encoded by the *Enod40* transcripts changes the plant cell response to auxin in tobacco protoplasts. An *Enod40* homologue was isolated from tobacco, and when expressed in tobacco protoplasts, had an activity identical to the legume Enod40, indicating that the peptides encoded by the *Enod40* genes may act as plant growth regulators (Crespi et al., 1996).

Several nodulin genes have also been identified during subsequent nodule organogenesis. One of these genes, the *Enod2* gene, was first isolated from soybean and pea (Franssen et al., 1987; van de Wiel et al., 1990a). The *Enod2* gene encodes a protein with a putative N-terminal signal peptide followed by a sequence containing repeating proline-rich

pentapeptide motifs (Franssen et al., 1987). *Enod2* homologues have been isolated from several other legumes, such as alfalfa (Dickstein et al., 1988), vetch (Moerman et al., 1987), *Sesbania* (Strittmatter et al., 1989; Dehio and de Bruijn, 1992), common bean (Sanchez et al., 1988), lupin (Szczyglowski and Legocki, 1992), and *Lotus japonicus* (R. Chen and F.J. de Bruijn, unpublished data). The *Enod2* mRNA is present in the inner cortex (nodule parenchyma) of determinate and indeterminate nodules (van de Wiel et al., 1990a), and in similar tissues in empty nodules induced by certain rhizobium strains (van de Wiel et al., 1990b). The exact function of the Enod2 protein has not yet been elucidated. However, since the nodule parenchyma appears to be a physical barrier limiting oxygen diffusion into the infected cells of nodules and the *Enod2* transcript (protein) is localized in this tissue, it has been postulated that this putative cell wall protein may contribute to the morphogenesis of the nodule parenchyma and therefore the generation of the oxygen diffusion barrier (Nap and Bisseling, 1990; van de Wiel et al., 1990a; Govers et al., 1990). One of the objectives of this dissertation research was to test this hypothesis by attempting to down-regulate *Enod2* gene expression using an antisense approach (see Chapter 5).

Following *Enod2* gene expression, the late nodulin genes are activated and nitrogen fixation commences. Late nodulins include enzymes involved in ammonia assimilation, such as glutamine synthetase and glutamate

synthase (GS, GOGAT, respectively; for reviews, see Atkins, 1987; Miflin and Lea, 1980); carbon metabolism, such as phosphoenolpyruvate carboxylase and sucrose synthase (PEP carboxylase, SS, respectively; for a review, see Atkins, 1987); and amide and ureide biogenesis, such as aspartate aminotransferase and uricase (AAT; uricase II; for a review, see Schubert, 1986). They also include proteins present in the peribacteroid membrane, such as Nodulin- 26 from soybean (Fortin et al., 1987; Miao and Verma, 1993; Weaver et al., 1994), leghemoglobins (Lbs; Appleby, 1984), and a putative plant transcription factor, NMH7 (Heard and Dunn, 1995). N-26 is a nodule-specific ion transporter (Weaver et al., 1994). Leghemoglobins (Lbs) function as oxygen carriers, facilitating O_2 diffusion to the nitrogen-fixing bacteroids (Appleby, 1984), and represent the most abundant proteins within nodules. Leghemoglobins are encoded by a multigene family, each member of which is induced at a slightly different time during nodule formation, indicating developmental control of *Lb* gene expression (Jensen et al., 1988). Different Lbs seem to have distinct affinities for oxygen, that may be important for nodule nitrogen fixing activity in subtle ways (Appleby, 1984; de Bruijn and Schell, 1992).

Late nodulin genes are the most well characterized nodule-specific genes, in terms of regulation of gene expression. Nodule-specific expression of the *Lb* genes, for example, has been studied in detail using reporter gene expression in transgenic plants (de Bruijn et al., 1990; Szabados et al.,

1990). Using deletions and specific mutations, an organ-specific *cis*-acting (OSE) element has been identified in the promoter region of a soybean leghemoglobin *lbc3* gene (Stougaard et al., 1987; Ramlov et al., 1993). Similarly, a nodule-infected-cell-specific (NICE) element, which contains three nodule conserved *cis*-elements (AAAGAT; TTGTCTCTT; and CACCCT), has been identified in the promoter region of a *Sesbania rostrata* leghemoglobin gene (*Srglb3*; Szabados et al., 1990; Szczyglowski et al., 1994).

D. Hormones as Secondary Messengers

As mentioned above, Nod factors stimulate multiple responses in legume plants, including root hair curling and deformation, cortical cell division, induction of nodulin gene expression and finally nodule formation. The molecular basis of Nod factor action is not precisely known. Ehrhardt et al. (1992) have shown that root hair membranes depolarize rapidly after addition of Nod factors. Allen et al. (1991) have found that cytoskeletal rearrangements, and changes in vacuole shape occur within root hair cells soon after the addition of Nod factors. Using calcium-sensitive reporter dyes, Ehrhardt et al., (1996) have shown that localized periodic spikes in cytoplasmic calcium levels occur following the application of Nod factors.

In plants forming indeterminate nodules, cortical cell divisions occur in the inner cortex, and Yang et al. (1994) have shown that only narrow rows of cortical cells located opposite protoxylem poles, are activated and express a histone *H4* gene. At the time of *H4* gene induction, the infection thread tips, the sites where Nod factors are released, are still in the epidermis. This indicates that Nod factors act at distance. Nod factors may directly activate the cortical cell division or indirectly affect them through endogenous signal molecules.

Plant hormones have been implicated in nodule development since Thimann (1936) first hypothesized that the plant growth hormone auxin was involved in pea root nodulation (for a recent review, see Hirsch, 1992). Subsequently, the plant hormone cytokinin was found to induce cortical cell divisions that resembled the initial response of roots to rhizobial infection (Arora et al., 1959; Libbenga and Bogers, 1974). Libbenga et al. (1973) determined that inner cortical cells opposite the xylem poles were stimulated to divide in devascularized explants after treatment with auxin and cytokinin. Furthermore, they found that an ethanol extract from the stele could replace cytokinin in promoting cell division. A substance responsible for this activity, the so-called stele factor (uridine), has since been isolated and is thought to be released from the protoxylem poles to promote susceptibility to the cortical cells located opposite the protoxylem poles (Smit et al., 1993; 1995).

More recently, both cytokinin (Cooper and Long, 1994) and compounds that block polar auxin transport (Hirsch, et al., 1989) have been shown to induce the formation of nodule-like structures in which several early nodulin genes, including *Enod2*, are expressed. Additionally, the Nod factor-inducible *ENOD40* gene has been shown to have plant hormone-like activity when expressed in alfalfa or tobacco (Crespi et al., 1994; van de Sande, 1996). It is possible that ENOD40 expression in the pericycle of legume roots causes a change in the cytokinin/auxin ratio in the cortical cells that triggers the initiation of cell division (Mylona et al., 1995).

Support for the notion that plant hormones play a role in nodule development also comes from studies which demonstrate that cytokinin can specifically induce early nodulin *Enod2* gene expression in the roots of *S. rostrata* (*SrEnod2*; Dehio and de Bruijn, 1992; Silver et al., 1996), and in roots of alfalfa (Hirsch et al., 1993). However, the mechanism by which cytokinin regulates early nodulin gene expression and the exact role of early nodulins in nodule development remain to be established.

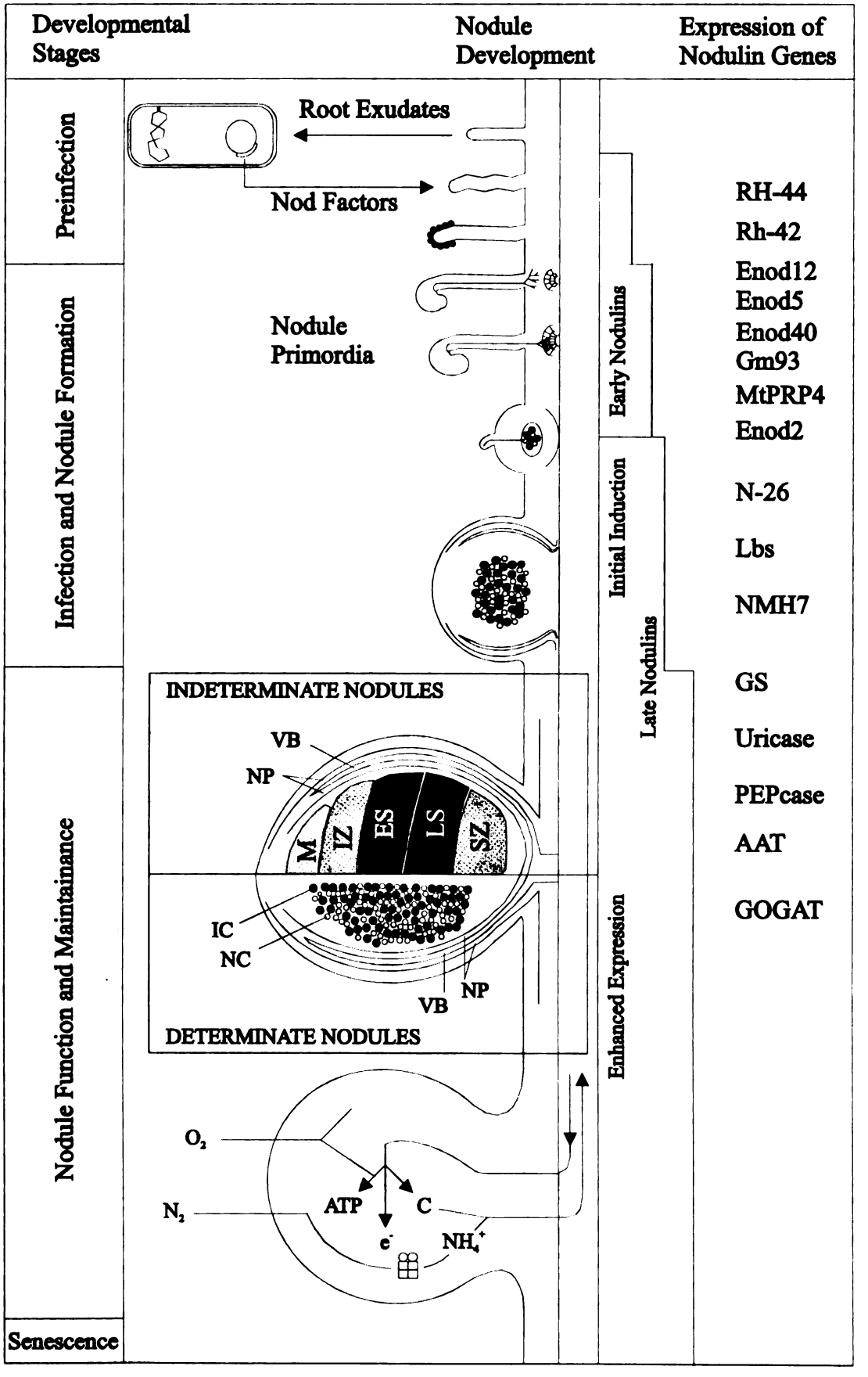
One objective of this dissertation research was to understand how the *SrEnod2* gene expression is controlled developmentally and cell specifically, and to identify *cis*-elements responsible for this type of regulation. Chapter 2 presents the results of this research. The data described in this chapter will be directly compared with those obtained in a separate study in our laboratory on the cytokinin regulation of *Enod2* gene

expression. One of the future goals will be to dissect the signal transduction pathways leading to the developmental, cell-specific, and cytokinin regulation of the *Enod2* gene expression by isolating *trans*-acting factors that interact with *cis*-elements identified here. Towards this goal, Chapter 3 describes some of the initial effort to identify putative protein factors interacting with these *cis*-elements using *in vitro* RNA-protein UV-cross linking strategies. To further our understanding of plant hormone regulation of *Enod2* gene expression, we also characterized the hormone response of the *Enod2* gene from *L. japonicus* (see Chapter 4). Surprisingly, we found that ethylene, instead of cytokinin, induces the *LjEnod2* gene expression, indicating that *Enod2* genes from different legume plants might be regulated differentially. Lastly, we employed a genetic approach in an attempt to down-regulate *Enod2* gene expression for the purpose of understanding the function of the *Enod2* protein during nodule development (see Chapter 5).

Since the results presented in Chapter 2 strongly suggest that the 3' untranslated region (3' UTR) of the *SrEnod2* gene mediates the developmental and cell-specific expression of this gene, in the following section, I will review the current literature on what is known about 3' UTR's in control of gene expression and developmental regulation.

LEGEND FOR FIGURE 1-1. Schematic representation of nodule developmental events and nodulin gene expression.

The developmental stages are shown on the left and the corresponding nodule development events are represented in the center. On the right, expression of some early and late nodulin genes is listed sequentially. Nodule types are represented in the middle (INDETERMINATE: M= meristem; IZ= infection; ES= early symbiotic; LS= late symbiotic; and SZ= senescing zones; VB= vascular bundle; NP= nodule parenchyma. DETERMINATE: IC= infected; NC= uninfected cells; VB= vascular bundle; NP= nodule parenchyma.). Major metabolic processes (bottom) are schematically represented as linked pathways of carbon, nitrogen, and oxygen metabolism. (Modified from Sandrez et al., 1991).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NP
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IZ
ES
LS
SZ
IC
NC
VB
NP
DETERMINATE NODULES
O2
N2
ATP
C
e-
NH4+
Early Nodulins
Initial Induction
Late Nodulins
Enhanced Expression
RH-44
Rh-42
Enod12
Enod5
Enod40
Gm93
MtPRP4
Enod2
N-26
Lbs
NMH7
GS
Uricase
PEPcase
AAT
GOGAT

SECTION II. Involvement of mRNA 3' Untranslated Regions (3' UTRs) in Control of Gene Expression and Developmental Regulation

Eukaryotic mRNAs, once transcribed in the nucleus, are processed, transported into the cytoplasm, bound by ribosomes, and translated. The accumulation of a particular mRNA is determined by the cumulative effects of its rates of synthesis and decay. Thus, post-transcriptional events such as mRNA turnover contribute a great deal to the regulation of gene expression. The time required for a mRNA to reach a new steady-state level, following a change in its rate of synthesis, has been shown to be directly proportional to its half-life (Hargrove and Schmidt, 1989). Therefore, the induction and repression of genes encoding unstable mRNAs are more rapid than those of genes encoding stable mRNAs. It has also been noted that protein and mRNA turnover are often coordinately regulated (Hargrove and Schmidt, 1989). This allows rapid regulation of steady-state protein levels after transcriptional activation of a particular gene. Control of mRNA turnover has been extensively studied in mammalian cells (for recent reviews, see Peltz et al., 1991; Ross, 1995, 1996; Decker and Parker, 1994), yeast (for a review, see Caponigro and Parker, 1996), and in plants (Green, 1993; Sullivan and Green, 1993; Abler and Green, 1996). Since in our studies, we found that the 3' UTR of the *SrEnod2* gene is responsible for the nodule

parenchyma-specific expression of this gene, this section will focus on 3' UTRs and their involvement in the processes that control gene expression. Of course, 3' UTRs are not the only sequences that are involved in the control of gene expression, and therefore other sequences that are important will also be briefly mentioned, wherever appropriate.

A. Instability Elements Localized in 3' UTRs

The half-lives of specific eukaryotic mRNAs vary over a large range. The basis for this difference is determined, to a large extent, by specific sequences within each mRNA. A number of elements that affect mRNA decay rates have been identified. One general class of elements consists of sequences that destabilize mRNAs. These instability elements have been identified in yeast, mammalian cells and plants. Instability elements have also been found in the 3' UTRs of many unstable mRNAs. Examples include the yeast *MFA2* (Muhlrad and Parker, 1992) mRNA; mammalian *c-fos* (Chen et al., 1994), *c-myc* (Jones and Cole, 1987), and granulocyte-macrophage colony-stimulating factor (GM-CSF; Shaw and Kamen, 1986) transcripts; as well as the plant SAUR (Small-Auxin-Up-RNA) transcript (Gil and Green, 1996).

The *MFA2* gene encodes the mating pheromone, a-factor, which needs to be rapidly removed from the cell after mating-type switching (Herrick et

al., 1990). Extensive molecular genetic analyses indicate that the 3' UTR contains sequences that specify rapid mRNA decay. Moreover, these sequences within the 3' UTR appear to be redundant, since various deletions and point mutations do not affect decay rate dramatically whereas double mutations or large deletions have a pronounced effect on slowing transcript decay (Muhlrad et al., 1992). Two repeating motifs, $Y_{6-8}CAU$ (where Y=C or U) and U(A/C)AUUUAUU, have been found to be involved in stimulating rapid mRNA deadenylation and subsequent decay.

Similar instability elements have been identified in the 3' UTRs of the *c-fos*, *c-myc* and GM-CSF transcripts. The mechanism by which these instability elements increase the rate of deadenylation and subsequent mRNA degradation is not known. However, the fact that these instability elements within 3' UTRs are often redundant and are located close to the poly(A) tail suggests that these sequences may provide multiple binding sites for factors that stimulate mRNA deadenylation, which is the first rate-limiting step in mRNA degradation of many eukaryotic mRNAs. Instability elements have also been identified in the coding regions of the *c-fos*, *c-myc* and many other unstable transcripts (Ross, 1995, 1996; Decker and Parker, 1994).

Other types of instability determinants have also been identified. For examples, the stem-loop structure at the 3' end of a cell cycle-regulated histone mRNA has been found to be necessary and sufficient for rapid

mRNA decay at the end of S phase (Pandey and Marzluff, 1987). The ion-responsive-elements (IREs) at the 3' UTR of the human transferrin receptor (hTR) gene have been found to be responsible for the iron-dependent feedback regulation of the transcript (Owen and Kühn, 1987). A long range stem-loop structure at the 3' UTR of mammalian insulin-like growth factor II (IGF-II) has been identified to be an endonuclease cleavage site and a mRNA stability determinant (Nielsen and Christiansen, 1992).

In plant cells, several different instability elements have been shown to affect half-life of transcripts. The first one is the highly conserved DST element, derived from the 3' UTRs of the unstable SAUR transcripts of soybean and other plants (McClure et al., 1989; Newman et al., 1993; for a review, see Green, 1993). It has been shown that a dimer of the DST sequences, when inserted into the 3' UTRs of two different reporter genes is sufficient to promote rapid degradation of the reporter transcripts in stably transformed tobacco cells, as well as in transgenic tobacco plants (Newman et al., 1993).

The second instability element found to destabilize plant gene transcripts is a synthetic sequence consisting of multiple copies of a AUUUA element (Ohme-Takagi et al., 1993), that is a repeating sequence found in the 3' UTRs of many unstable mammalian transcripts, including *c-fos* and GM-CSF (Peltz et al., 1991). Insertion of this sequence into the 3'

UTRs of reporter genes stimulates rapid degradation of the reporter transcripts in stably transformed tobacco cells (Ohme-Takagi et al., 1993).

B. Stability Elements Localized in 3' UTRs

It is known that mRNA instability elements are responsible for rapid decay of unstable transcripts. However, it is unclear whether stable transcripts contain stability determinants or are stable by default. Recently, the 3' UTR of the human α2-globin gene has been found to contain erythroid cell-specific mRNA stability elements (Weiss and Liebhaber, 1995), indicating that at least some stable transcripts contain stability elements. A base-pair scanning mutagenesis in the 3' UTR of the α2-globin gene has revealed that three C-rich regions are required for the longevity of the α2-globin gene in erythroid cells. Mutations that allow the translating ribosome to read through into the α2-globin 3' untranslated region target the mutant mRNAs for accelerated turnover in erythroid cells, but not in non-erythroid cells, indicating a cell type-specific half-life regulation of this transcript (Weiss and Liebhaber, 1994).

C. Messenger RNA Intracellular Localization Signals in 3' UTRs

Although very little is known about intracellular localization of mRNAs in plants, a lot of information is available for mammalian systems. It has been proposed that intracellular localization of specific maternal mRNAs is a general mechanism for protein targeting that probably occurs in all polarized cell types (see St Johnston, 1995). Localization of mRNAs allows specific proteins to be synthesized in the sub-cellular location where they are required and prevents their expression in areas where they are not.

In *Drosophila melanogaster* oocytes, several mRNAs have been found to be localized to different locations, and these transcripts play a variety of roles in the specification of the embryonic body plan (see Ding and Lipshitz, 1993). For examples, the *oskar* mRNA localizes to the posterior pole and plays a role in defining anteroposterior polarity by determining where the pole plasm forms (Ephrussi and Lehmann, 1992). Similarly, *gurken* mRNA localizes to the dorsal anterior corner of the oocyte and defines the dorsoventral axis of the embryo, by being translated to produce a transforming growth factor α (TGFα)-like protein (Neuman-Silberberg and Schüpbach, 1993). On the other hand, *bicoid* and *nanos* mRNAs are anchored at the anterior and posterior poles, respectively, and act as localized sources of diffusible proteins that spread toward the center of the embryo. The resulting opposite protein gradients specify the pattern and

polarity of the embryo (St Johnston and Nusslein-Volhard, 1992). In *Xenopus laevis* oocytes, like in its *Drosophila* counterpart, maternal messengers are also localized to different sites (Kloc et al., 1993).

A common feature of all the localized mRNAs identified so far is that the *cis*-acting sequences required for localization reside in the 3' UTRs of transcripts and are typically long. One of the best characterized localization signal derives from the *bicoid* mRNA. It is 625 nt long and located within the *bicoid* 3' UTR, of which a 53 base sequence, called the BLE1 element, is required and sufficient to direct localization of the *bicoid* mRNA to the anterior of the oocyte (Macdonald et al., 1993). A 400 nt region overlapping the BLE1 elements is required for the anchoring of the mRNA in the anterior cytoplasm, once released there. The latter step requires an association of the 400 nt sequence with the staufen protein to prevent diffusion of the RNA (Ferrandon et al., 1994). Therefore, the *bicoid* localization signal contains at least two distinct, but overlapping, sequence elements that mediate different steps in its localization.

Interestingly, the same staufen protein is also required for the translocation of *oskar* mRNA from anterior to posterior of the oocyte. The localization of the staufen protein switches from the posterior pole to the anterior pole soon after fertilization, thus facilitating translocation of two differently localized transcripts (Ferrandon et al., 1994). The Staufen protein contains five copies of a dsRNA-binding motif and binds to dsRNA

in vitro (St Johnston et al., 1992). Since the binding is not sequence-specific *in vitro*, it is possible that staufen recognizes the secondary structure of *bicoid* and *oskar* RNAs, rather than their sequence (St Johnston et al., 1992).

Several other mechanisms responsible for RNA localization within a cell have been identified. One of them uses spatial control of mRNA stability. This is a simple but inefficient way to localize RNA. The localization of *hsp83* RNA to the posterior pole of the *Drosophila* embryo involves a localized protection from mRNA degradation in the posterior pole plasm (Ding et al., 1993). On the other hand, the localization of *hunchback* RNA in the anterior involves a localized destabilization in the posterior due to a translational repression of the *hunchbach* RNA by *nanos* (Curtis et al., 1995).

D. Messenger RNA 3'-End Formation

In eukaryotic cells, mRNA 3'-end formation is an important process in the regulation of gene expression. This process includes cleavage of transcripts and addition of a tract of poly(A). In general, polyadenylated RNAs are more stable than their unpolyadenylated counterparts. The mechanism that governs the 3'-end formation of mammalian mRNAs has been well characterized (Proudfoot, 1991; Wahle et al., 1992). The hexanucleotide

AAUAAA, the highly conserved poly(A) signal, is located 10-30 nt upstream of the poly(A) site. In addition to the poly(A) signal, a less conserved downstream GU-rich sequence is also required for efficient 3'-end formation. At least four protein complexes are involved in the 3'-end processing. The cleavage polyadenylation-specific factor (CPSF) recognizes the poly(A) signal, while the cleavage stimulation factor (CstF) recognizes the downstream GU-rich sequence. The cooperative binding of these two factors to the precursor mRNA brings in cleavage factors (Cfs) and poly(A) polymerase (PAP) for the cleavage and polyadenylation of the precursor mRNA, respectively.

The mechanism that controls the 3'-end formation of transcripts encoded by plant nuclear genes has started to be elucidated (for reviews, see Hunt, 1994; Wu et al., 1995). It is apparent that the poly(A) signal is not conserved near poly(A) sites in many plant nuclear genes. A systematic search for the AAUAAA signal near poly(A) sites indicates that about 1/3 of genes surveyed contain the AAUAAA sequence, about 1/2 of genes contain 4-5 of 6 base pair matches for this sequence and about 15% of genes do not have any AAUAAA-like sequence (Joshi, 1987). Additionally, the 3' untranslated regions (3'UTRs) of most plant nuclear genes tend to contain multiple AATAAA-like and GT-rich sequences.

Unlike their mammalian counterparts that require downstream GU-rich sequence for efficient 3'-end processing, most plant mRNAs require less

conserved, upstream sequences (Hunt, 1994). Removal of these sequences results in a drastic decrease in polyadenylation. In some plant mRNAs, downstream regions are required for efficient polyadenylation. These include *rbcS* and *ocs* mRNAs (Hunt and MacDonald, 1989; Macdonald et al., 1991). Using a functional analysis, An et al. (1989), have demonstrated that the 3' flanking region of the potato wound-inducible proteinase inhibitor II gene that contains the poly(A) signal and a downstream GT-rich sequence is essential for wound-inducible reporter gene expression.

E. Polyadenylation and Translational Control of Gene Expression

Controlling gene expression by regulating translation allows a cell to respond to environmental cues more quickly than *de novo* transcription permits. Translation can be regulated by modulating several factors, including translation initiation and the length of the poly(A) tail. Since ribosomal binding is most sensitive to secondary structures in the 5' UTR, the sequence of the 5' UTR determines the intrinsic rate of translation initiation of an mRNA. However, *in vivo* and *in vitro* evidence support the idea that the poly(A) tail stimulates the rate of translational initiation of a mRNA. Recently a yeast poly(A) binding protein (Pab1p)- poly(A) complex has been found to recruit the 40S ribosomal subunit to the mRNA, indicating that the 5' and 3' ends of a mRNA physically interact (Tarun and

Sachs, 1995). Recently, poly(A) tails of most mammalian mRNAs have been found to be modulated in cytoplasm by cytoplasmic polyadenylation machinery (Simon et al., 1992). Cytoplasmic polyadenylation requires two sequence elements within 3' UTRs: a U-rich cytoplasmic polyadenylation element (CPE) and the nuclear polyadenylation sequence AAUAAA (Wormington, 1993). Precise spacing of a CPE element relative to the AAUAAA sequence has been found to be important for developmental control of polyadenylation (Simon et al., 1992). Other specific translational regulatory sequences distant from the CPEs have been identified in the 3' UTRs of a number of developmentally regulatory genes. One of them is the 10 random repeats of a pyrimidine-rich motif (R sequence) located in the 3' UTR of the *Lox* gene, which encodes the 15-lipoxygenase enzyme required for the breakdown of internal membranes in mature reticulocytes (Ostareck-Lederer et al., 1994). A 48 KDa protein has been identified to specifically bind to these repeats and this binding is necessary and sufficient for translational repression of a heterologous mRNA containing the R sequences in an *in vitro* assay (Ostareck-Lederer et al., 1994).

F. A Regulatory Role for 3' UTRs in Growth and Differentiation

Using a genetic complementation approach, three genes, troponin I, tropomyosin, and a-cardiac actin, have been identified that activate muscle-

specific promoters in a differentiation-deficient mutant (NMU2) (Rastinejad and Blau, 1993). This activity was mapped to the 3' UTRs of these genes and was found not to be limited to myogenic cells, since the 3' UTRs also promoted differentiation of wild type muscle cells, and suppressed proliferation of fibroblasts (Rastinejad and Blau, 1993). The mechanism by which these 3' UTRs promote differentiation and inhibit growth is not known. However, it is speculated that 3' UTRs of certain differentiation-specific RNAs are *trans*-acting regulators that modulate cell division and therefore differentiation. The phenomenon that 3' UTRs modulate growth and differentiation has been further exemplified by the observation that the 3' UTR of tropomyosin can efficiently suppress mouse rhabdomyosarcomas, induced by transplanting the NMU2 cell line into muscle cells of adult mouse hindlimbs (Rastinejad et al., 1993).

G. SUMMARY

Symbiotic nitrogen fixation takes place in the infected cells of legume nodules. This process requires a large input of energy and limitation of the oxygen concentration, presenting the oxygen paradox. Legume nodules have developed strategies, such as formation of diffusion barriers, production of a large amount of the oxygen carrier (leghemoglobin), and active respiration of bacteroids. A further detailed understanding of how

these various processes are coordinated during nitrogen fixation will be necessary before one could even contemplate transferring the symbiotic nitrogen-fixation system to non-nodulated agronomically important crop plants, such as rice (de Bruijn et al., 1995).

Nodule organogenesis is triggered by Nod factors. Nod factors are hypothesized as plant growth regulators, since they cause root hair deformation and stimulate cell divisions. Nodule organogenesis provides a model to study signal molecules, their perception and action. Nodules provide a rich source of specifically induced plant and bacterial genes to study gene regulation. Some of these genes are regulated by Nod factors and plant hormones. They may prove to serve as excellent tools to study the corresponding signal transduction pathways and this, in turn, will help to understand how nodule organogenesis utilizes and integrates these various pathways.

Regulation of gene expression occurs at different levels. Post-transcriptional regulation is clearly very important for modulating gene expression. It allows a cell to rapidly adjust gene expression to accommodate developmental and environmental cues. This type of regulation occurs both in the nucleus and cytoplasm. Steps involved include 5' capping and 3' polyadenylation in the nucleus, transport to cytoplasm, poly(A) tail shortening and lengthening in the cytoplasm, degradation and stabilization, intracellular localization, and translation. Each step involves

the recognition of *cis*-elements and their corresponding *trans*-acting factors. Some of these steps are connected and coordinately regulated. 3' untranslated regions of mRNAs have been implicated in various processes of gene regulation and 3' UTRs of some structural genes have been found to exert profound effects on growth and differentiation by acting as *trans*-acting regulators (riboregulators). In this dissertation research (chapter 2), I have identified the 3' UTR of the early nodulin *SrEnod2* gene as the mediator for the tissue-specific expression. To our knowledge, this type of regulation is the first example among plant nuclear genes clearly demonstrating that the 3' UTR regulates tissue-specific expression.

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CHAPTER 2

THE NODULE PARENCHYMA-SPECIFIC EXPRESSION OF THE *SESBANIA ROSTRATA* EARLY NODULIN GENE *SrENOD2* IS MEDIATED BY ITS 3' UNTRANSLATED REGION (3'UTR)

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ABSTRACT

The early nodulin *Enod2* gene encodes a putative hydroxyproline-rich cell wall protein, and is expressed exclusively in the nodule parenchyma cell layer. The latter suggests that the Enod2 protein may contribute to the special morphological features of the nodule parenchyma and to the creation of an oxygen diffusion barrier (Nap and Bisseling, 1990, Science 250, 948-954). We have shown that the *Enod2* gene of the stem-nodulating legume *Sesbania rostrata* (*SrEnod2*) can be specifically induced in roots by the plant hormone cytokinin (Dehio and de Bruijn, 1992, Plant J. 2, 117-128), and that this induction occurs at a posttranscriptional level (Silver et al., 1996, Plant Physiol. 112, 559-567). Here, we have characterized *cis*-determinant(s) in the *SrEnod2* locus responsible for nodule parenchyma-specific expression and report that the 3' untranslated region (3' UTR) of the *SrEnod2* gene is both required and sufficient for directing chimeric reporter gene expression in the nodule parenchyma of transgenic *Lotus corniculatus* plants. By carrying out a detailed deletion analysis of the 5' and 3' *SrEnod2* regions, we delimited the minimal promoter of the *SrEnod2* gene and revealed that the 5' flanking sequences do not appear to be essential for nodule parenchyma-specific expression. This finding contrasts with the report that the 5' upstream region of the soybean *Enod2* gene directs nodule parenchyma-specific expression (Lauridsen et al., 1993, Plant J. 3, 483-

492), indicating different mechanisms may be involved in regulation of the expression of these two genes. To our knowledge, this is also the first demonstration that *cis*-element(s) specific for tissue-specific expression are located within the 3' UTR of a plant nuclear gene.

INTRODUCTION

The *Enod2* gene is an early nodulin gene that was first identified by differential screening of a soybean root nodule cDNA library (Franssen et al., 1987). Isolation of *Enod2* homologues from other legumes, and subsequent DNA sequence comparisons, have shown that the *Enod2* gene is highly conserved among leguminous plants (Franssen et al., 1987; Dickstein et al., 1988; Govers et al., 1990; van de Wiel et al., 1990a; Szczyglowski and Legocki, 1990; Dehio and de Bruijn, 1992). The amino acid sequence derived from the soybean *Enod2* cDNA clone revealed that the Enod2 protein is very proline-rich and is mainly composed of two repeating pentapeptides, ProProHisGluLys and ProProGluTyrGln (Franssen et al., 1987; van de Wiel et al., 1990a). The Enod2 proteins of other legumes appear to be composed of the same or similar repeating pentapeptides (Dickstein et al., 1988; van de Wiel et al., 1990a; Szczyglowski and Legocki, 1990; Dehio and de Bruijn 1992). A putative signal peptide is present at the N-terminus of all of the Enod2 proteins identified thus far,

and the amino acid sequences of both pea and soybean Enod2 proteins highly resemble a hydroxyproline-rich cell-wall protein identified in soybean seed cell walls (Averyhart-Fullard et al., 1988; van de Wiel, et al., 1990a). Therefore, it has been proposed that the Enod2 protein is a cell wall protein (van de Wiel et al., 1990a).

The *Enod2* gene is expressed during early stages of nodule organogenesis (Franssen et al., 1987; van de Wiel et al., 1990a; Dehio and de Bruijn 1992). It has been shown that this early nodulin gene is also expressed in empty nodules induced by certain *Rhizobium* strains on the roots of soybean and alfalfa (Finan et al., 1985; Franssen et al., 1987; Dickstein et al., 1988). These empty nodules contain neither infection threads nor intracellular bacteroids. Expression of the *Enod2* gene has also been demonstrated in "pseudo-nodules" induced by auxin transport inhibitors (Hirsch et al., 1989; van de Wiel et al., 1990b) or by *Rhizobium* nodulation-deficient (Nod^-) mutants secreting the cytokinin *trans*-zeatin (Cooper and Long, 1994). The expression of the *Enod2* gene in these empty/pseudo-nodules strongly suggests that the Enod2 protein is not involved in the infection process *per se*, but may play a role in nodule ontogeny.

The finding that the *Enod2* gene is expressed in alfalfa pseudo-nodules induced by auxin transport inhibitors (Hirsch et al., 1989) extends the observations made several decades ago suggesting that plant hormones are

involved in the induction of nodules on plant roots (Thimann, 1936; Arora et al., 1959; Libbenga et al., 1973; reviewed by Hirsch, 1992; Verma, 1992). In fact, plant hormones clearly appear to be involved in the expression of the *Enod2* gene; Dehio and de Bruijn (1992) first demonstrated that the expression of the *Enod2* gene of *Sesbania rostrata* is specifically up-regulated in roots after the application of cytokinin. Similar results have been reported for the alfalfa *Enod2* gene (Hirsch et al., 1993), although the size of the cytokinin-enhanced *Enod2* transcript in roots is larger than that observed in alfalfa nodules.

In-situ hybridization studies have demonstrated that the soybean *Enod2* gene is expressed in the nodule parenchyma and in the nodule tissue surrounding the vascular bundle that connects the nodule to the root central cylinder (van de Wiel et al., 1990a). Low levels of the *Enod2* transcript have also been found in the nodule endodermis and the nodule outer-cortical cells directly adjacent to the endodermis (van de Wiel et al., 1990a). The nodule parenchyma is composed of several layers of cells, with fewer and smaller intercellular spaces than most other cortical cells (van de Wiel et al., 1990a). The compact structure of these cell layers has suggested to various investigators that they may be involved in forming an oxygen diffusion barrier (Tjepkema and Yocum, 1974; Witty et al., 1986; Nap and Bisseling, 1990; van de Wiel et al., 1990a). Since the Enod2 protein is localized in the same cell layers, it has been hypothesized to contribute to the special

morphology of the nodule parenchyma and therefore to the formation of the oxygen barrier (Nap and Bisseling, 1990; van de Wiel et al., 1990a). However, no experimental evidence supporting this hypothesis exists.

Recently, considerable efforts have been made to understand how *Enod2* gene expression is controlled in developing soybean and *S. rostrata* nodules and in roots of *S. rostrata* plants after cytokinin treatment (Lauridsen et al., 1993; de Bruijn et al., 1994; Silver et al., 1996). We have shown that the cytokinin-mediated up-regulation of the *SrEnod2* gene in uninfected roots of *S. rostrata* occurs mainly in cytoplasm at the post-transcriptional level, requires protein synthesis, and appears to involve protein phosphorylation (Silver et al., 1996). We have also reported that a 3-kb 5' upstream region of the *SrEnod2* gene failed to consistently direct the expression of the *uidA* reporter gene (*gus;* Jefferson et al., 1987) in the nodule parenchyma of transgenic *Lotus corniculatus* plants (de Bruijn et al., 1994). In contrast, a 3-kb 5' region of the soybean *Enod2(B)* gene [*GmEnod2(B)*] has been shown to direct reporter gene expression in the nodule parenchyma of transgenic *L. corniculatus* plants (Lauridsen et al., 1993). Here, we describe the detailed analysis of DNA sequence determinants in the *SrEnod2* locus responsible for tissue-specific expression in nodules. We conclude from this analysis that, although the 5' proximal region of the *SrEnod2* gene is required for gene expression, the 3' untranslated region (3' UTR) of the *SrEnod2* gene contains DNA sequence

determinants responsible and sufficient for nodule parenchyma-specific gene expression.

METHODS

Plant growth and nodulation

Sesbania rostrata seeds were surface-sterilized and germinated on petri dishes in the dark at 28°C for one day, as described by Pawlowski et al., (1991). Seedlings were grown for three weeks in autoclaved soil mix (MetroMix:sand; 2:1) in growth chambers with an 18-hr light (28°C) and 6-hr dark (22°C) cycle. Plants were inoculated with a two-day-old culture of *Azorhizobium caulinodans* ORS571 (Dreyfus and Dommergues, 1981) either in pots for root nodulation or on stems for stem nodulation. Six-day-old root nodules and stem nodules were collected and processed (see below).

Lotus corniculatus transgenic plants were grown for one week in autoclaved soil mix (vermiculite:sand:metromix; 10:10:1) in growth chambers with a 16-hr light (24°C) and 8-hr dark (18°C) cycle. Plants were inoculated with a three-day-old culture of *Rhizobium loti* NZP2037 (Pankhurst, 1983) and grown for 21 days. Plant tissues (nodules, infected roots, and leaves plus stems) were collected and stored in liquid nitrogen.

In-situ RNA localization

In-situ RNA localization studies were carried out essentially as described by Cox and Goldberg (1988) and Van de Wiel et al. (1990a), except that a non-radioactive approach was used according to De Block and Debrouwer (1993). Stem and root nodules were fixed in a 10 mM sodium phosphate buffer (pH 6.8) containing 4% paraformaldehyde, 0.25% glutaraldehyde, and 100 mM NaCl at room temperature, dehydrated in a graded ethanol and xylene series, and embedded in paraplast X-tra (Fisher Scientific, Fair Lawn, NJ). Nodule sections (7 μm thick) were cut and attached to superfrost/plus microscopic slides (Fisher Scientific, Pittsburgh, PA). After removal of the paraplast with xylene and rehydration through an ethanol series, sections were treated with proteinase K (5 μg/ml) in 100 mM Tris-HCl (pH 7.5); 50 mM EDTA for 30 min at 37^{o}C and with 0.25% acetic anhydride in 100 mM triethanolamine (pH 8.0) for 10 min at room temperature. Slides were subsequently dehydrated through an ethanol series and dried and hybridized with digoxigenin-labeled sense and antisense RNA probes generated by transcribing the coding region of the *SrEnod2* gene (Dehio and de Bruijn, 1992) with T3 and T7 RNA polymerase, according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany).

Hybridization and washing were essentially carried out according to Cox and Goldberg (1988) and De Block and Debrouwer (1993). Sections were dehydrated via an ethanol series and mounted with poly-mount (Polysciences Inc, Warrington, PA). Sections were photographed using an Axiophot microscope (Zeiss, Oberkochen, Germany).

Isolation of total RNA and poly(A)$^+$ RNA

Total RNA was isolated according to the hot phenol method of Verwoerd et al., (1989), with the following modifications. Briefly, plant material (up to 1 gm) was ground in liquid nitrogen to a fine powder and then transferred into a 15-ml tube containing 4 ml of preheated (65^{o}C) RNA isolation buffer (Hall et al., 1978), and 4 ml of preheated (65^{o}C) phenol. Samples were vortexed vigorously for 5 min, 4 ml of chloroform was added, the mixture was vortexed again for 15 min and centrifuged at 10,000 rpm for 20 min. The supernatant was extracted once with an equal volume of chloroform and centrifuged again. RNA in the supernatant was precipitated at 10,000 rpm for 30 min after adding 1/4 volume of 8 M LiCl and 1/10 volume of 1.6 M KCl and an overnight incubation on ice. The RNA pellet was resuspended in 400 µl of DEPC-treated water and extracted with an equal volume of chloroform until a clear interface was formed. RNA was then

precipitated and resuspended in DEPC-H_2O. The RNA concentration was determined photometrically.

Poly(A)$^+$ RNA was prepared by passing total RNA through oligo-dT columns according to the manufacturer's instructions (5 prime→3 prime, Boulder, CO). Northern analysis was performed according to standard procedures (Maniatis et al., 1982). Briefly, Poly(A)$^+$ RNA was loaded on a 1% formaldehyde agarose gel and separated at 4^{o}C. The gel was soaked in 10x SSC for 30 min, to remove formaldehyde, and subsequently blotted to a 0.22 μm nitrocellulose membrane (NitroPlus, Micron Separation Inc, Westborough, MA). The membrane was rinsed briefly in DEPC-H_2O and baked at 80^{o}C for 2 hr under vacuum. Prehybridization was carried out at 51^{o}C overnight in a solution containing 5x SSC, 10x Denhardt's solution, 0.1% SDS, 0.1M KPO_4, pH 6.8, and 100 μg/ml salmon sperm (ss) DNA. Hybridization reactions were carried out at 65^{o}C for 18 hr in a solution containing 1x SSC, 10x Denhardt's solution, 0.1M KPO_4 (pH 6.8), 100μg/ml ssDNA, 10% dextran sulphate, 50% formamide. After hybridization, the membrane was washed at 65^{o}C in 2x SSC and 1x SSC for 20 min each and finally in 0.2x SSC for 40 min. Blots were exposed to X-ray film at -75^{o}C.

Chimeric reporter gene construction

A 5.3-kb EcoRI fragment, which contains a 1.9-kb 5' region, a 1-kb coding region, and a 2.4-kb 3' downstream region of the *SrEnod2* gene was subcloned from the genomic clone λCD1 (Dehio and de Bruijn, 1992) into the pBluescript KS- vector (Stratagene, La Jolla, CA). Restriction sites (BamHI and SalI) were introduced at the translational initiation codon and at the 3' end of the putative signal peptide sequence of the *SrEnod2* gene, using site-directed mutagenesis as described by Kunkel et al. (1985). The oligonucleotide primers used for introducing the BamHI and SalI sites were (5')GAGTAGTGTAGAGAGGATCCTTCTATCTAT(3') and (5')GGCTCA TAGTAATTAGTCGACACTGGAG(3'), respectively. The underlined nucleotide sequences correspond to the restriction endonuclease recognition sites and the highlighted nucleotides correspond to the mutations introduced. A 1.9-kb EcoRI-BamHI fragment, which contains the entire 5' untranslated region and upstream region of the *SrEnod2* gene, was inserted in front of the *gus* coding region carried on plasmid pBI101.1 (Clontech Laboratories, Palo Alto, CA). The EcoRI end of the fragment was filled in by Klenow before cloning. The resulting binary vector was used to generate constructs 1 and 2 (Figure 2-2A). Similarly, a 1.98-kb EcoRI-SalI fragment, which contains the 75-bp signal peptide sequences in addition to the 5' sequences of the *SrEnod2* gene, was inserted in front of the *gus* coding

region of plasmid pBI101.2, and the resulting vector was used to generate construct 5. To construct a transcriptional fusion between the 1.98-kb 5' sequences of the *SrEnod2* gene and the *gus* coding region, a second-round mutagenesis was performed on the 5.3-kb EcoRI fragment containing the introduced SalI site with an oligonucleotide carrying a mutation in the translational initiation codon (ATG to GGA). The resulting 1.98-kb EcoRI-(BamHI)-SalI fragment was excised and inserted in front of the *gus* coding region carried on plasmid pBI101.1 This vector was used to generate constructs 3 and 4 (Figure 2-2A).

A restriction site (SacI) was introduced immediately downstream of the translational stop codon (TAA) of the *SrEnod2* gene using the oligonucleotide primer (5')GTAGTGGTAGTGGTTG**GAGCTC**TTAATTT TTTTTGG(3'). A 2.4-kb SacI-EcoRI fragment, containing the entire 3' untranslated and downstream region of the *SrEnod2* gene, was inserted into pBI101.1 to generate constructs 1, 3, and 5 (Figure 2-2A).

Constructs 6 and 7 were generated by inserting a 3.4-kb HindIII-SacI fragment of plasmid pLP14 (Szczyglowski et al., 1994), containing a 1.4-kb 5' sequence of the *Srglb3* gene and the *gus* coding region, into pBI101.1 and pBI101-3'*SrEnod2*, respectively. Constructs 8, 9, 10, and 11 were generated in a similar fashion by inserting a 2.1-kb BamHI-SacI fragment of pLP70 containing the 96-bp minimal CaMV35S promoter (-90 bp to +6 bp) and the *gus* coding region; a 2.15-kb BamHI-SacI fragment of pLP62,

containing the 150-bp minimal nopaline synthase promoter (-150 bp to +1 bp) and the *gus* coding region, into the BamHI-SacI sites of pBI101.1 and pBI101-3'*SrEnod2*, respectively.

Several 5' deletion fragments were generated by restriction enzyme digestion of the 1.9-kb EcoRI-BamHI fragment. XbaI, Sau3A, RsaI, EcoRV, DraI, MspI HinfI, AluI, SspI and Tsp were used to generate deletions ending at positions -1628, -1180, -854, -684, -464, -384, -306, -191, -115, and -50 bp, relative to the transcriptional start point. Several 3' deletion fragments were generated by nested deletions using the Erase-a-Base kit (Promega, Madison, WI). A XhoI linker was inserted into the EcoRI site at the end of the 3' sequences of the *SrEnod2* gene in construct 1. The 3' deletion fragments were then inserted into the SacI-XhoI sites of the modified construct 1.

Regeneration of transgenic *Lotus corniculatus* plants

Transgenic *Lotus corniculatus* cv *Rodeo* plants were generated according to Szabados et al. (1990). Binary vectors were conjugally transferred into the *Agrobacterium rhizogenes* strain A4 (Tempe and Casse-Delbart, 1989). Regenerated plants were transferred to a growth chamber (Conviron, Asheville, NC) and nodulated as described (Szabados et al., 1990).

Quantification of GUS enzymatic activity

GUS enzymatic activity was quantified according to Jefferson et al. (1987). The units of GUS activity used were picomoles of 4-methylumbelliferone (MU) produced *per* minute *per* milligram of protein. The concentration of protein in the extract was determined by the Bradford assay (Bradford, 1976). Fluorometric assays were performed using a fluorescence spectrophotometer (Model F-2000; Hitachi, Tokyo, Japan).

Histochemical staining of GUS enzymatic activity

GUS enzymatic activity was analyzed histochemically according to the procedure described by Jefferson (1987), with the following modifications. Hand-cut nodule sections were incubated at 37^{o} in a solution containing 50 mM Na_2PO_4 and $NaHPO_4$, pH 7, 1 mM ferri-/ferro-cyanide and 20% methanol and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide; Gold Biotechnology Inc., St. Louis, MO). Stained sections were then embedded in historesin (Reichert-Jung; Cambridge Instruments, Heidelberg, Germany), as described by De Block and DeBrouwer (1992), or in paraplast X-Tra, as described by Cox and Goldberg (1988). Sections of 10 μm-thick were generated and examined by dark- and light-field microscopy using an Axiophot microscope.

RESULTS

Localization of the *SrEnod2* transcript in the parenchyma of *S. rostrata* stem and root nodules

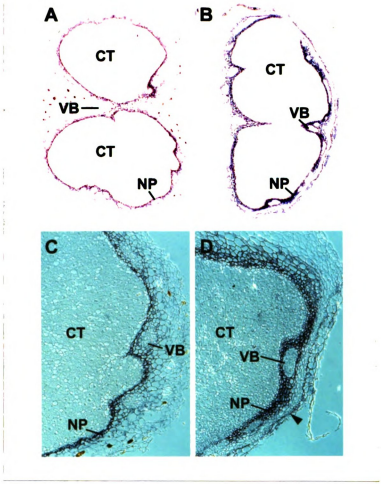
The tropical legume *S. rostrata* forms nodules on both roots and stems after infection with *Azorhizobium caulinodans* ORS571 (Dreyfus and Dommergues, 1981; de Bruijn, 1989). The formation of stem nodules is induced following a crack-entry mediated infection of *A. caulinodans* ORS571 at the base of dormant root primordia, which are present in rows along the stem (Tsien et al., 1983; de Bruijn, 1989). Root nodule organogenesis in *S. rostrata* appears to be intermediate between the indeterminate and determinate processes of nodule formation (Ndoye et al., 1994). These observations suggest that nodule formation in *S. rostrata*, as in many other tropical legumes, represents a primitive type of nodule organogenesis (Ndoye et al., 1994), and prompted us to study the tissue-specific expression of the *SrEnod2* gene in *S. rostrata* stem and root nodules. The results, presented in Figure 2-1, show that the *SrEnod2* gene is expressed in the nodule parenchyma cells of both stem and root nodules (Figure 2-1A and B). In both types of nodules, hybridization signals are also observed in cells immediately adjacent to the nodule vascular tissue (Figure 2-1C and D). Moreover, in stem nodules, hybridization signals are also

LEGEND FOR FIGURE 2-1. In-situ localization of *SrEnod2* gene expression.

Sections (7-μm) of *S. rostrata* root and stem nodules harvested 6 days after inoculation with *A. caulinodans* ORS571, were hybridized to a digoxigenin-labeled antisense RNA probe derived from the *SrEnod2* coding region. Pink and black colors indicate hybridization signals. No signals were observed in neighboring sections hybridized with the sense probe (data not shown).

(A) Section of a root nodule.
(B) Section of a stem nodule.
(C) Magnification of the section shown in (A).
(D) Magnification of the section shown in (B).

CT, central tissue; NP, nodule parenchyma; VB, vascular bundle. The arrowhead indicates expression in the nodule endodermis.


A
CT
VB
CT
NP
B
CT
VB
NP
C
CT
VB
NP
D
CT
VB
NP

detected in cell layers corresponding to the nodule endodermis (Figure 2-1D, arrowhead). Expression of the *SrEnod2* gene was not observed in other nodule cells. Therefore, the expression pattern of the *SrEnod2* gene in the parenchyma cells of *S. rostrata* stem and root nodules appears to be similar to that of the *Enod2* genes of other legumes (van de Wiel et al., 1990a).

The 3' flanking region of the *SrEnod2* gene is required for gene expression in the nodule parenchyma

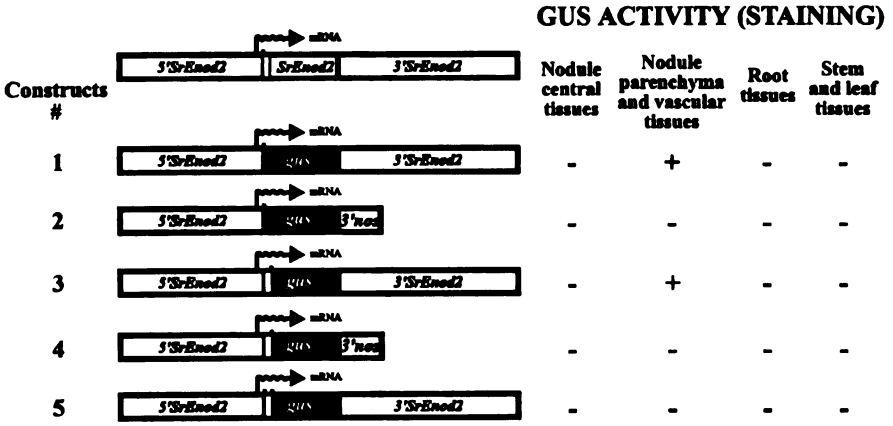
To identify DNA sequence determinants responsible for nodule parenchyma-specific expression of the *SrEnod2* gene, a chimeric reporter gene fusion (*gus*; Jefferson, 1987; Jefferson et al., 1987) was constructed, in which the 5' and 3' flanking regions of the *SrEnod2* gene were connected to the coding region of the *gus* reporter gene (5'*SrEnod2*-*gus*-3'*SrEnod2*; Figure 2-2A, construct 1). A construct containing the 3' terminator region of the nopaline synthase gene (3'*nos*) was used as a control (5'*SrEnod2*-*gus*-3'*nos*; Figure 2-2A, construct 2). These two constructs were introduced into *L. corniculatus* plants by *Agrobacterium rhizogenes*-mediated plant transformation (Szabados et al., 1990), and their presence in transgenic plants was verified by Southern blotting (data not shown). Expression of the

LEGEND FOR FIGURE 2-2. Structure of *gus* fusion constructs and determination of GUS activity in transgenic *L. corniculatus* plants.

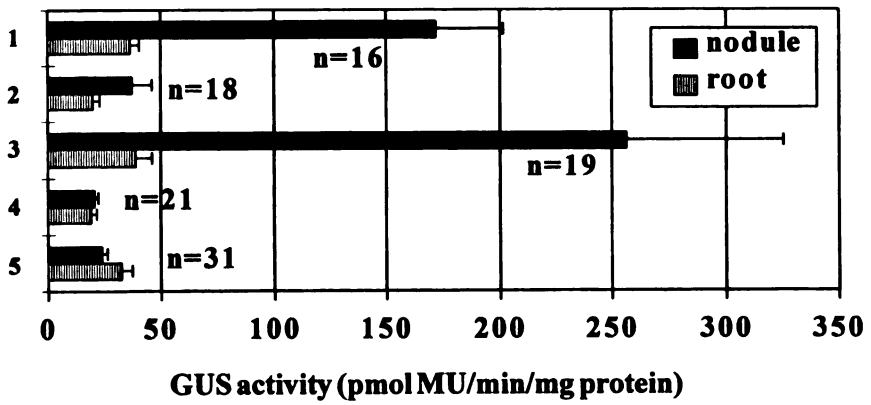
(A) Structure of *gus* fusion constructs. The construct numbers are listed. The boxes in black and white represent *gus*, *SrEnod2* and *nos* gene sequences, respectively. The thin open box represents a 75-bp putative signal peptide sequence. The vertical lines and wavy arrows represent the transcriptional start site of the *SrEnod2* gene. The vertical bars attached to the boxes represent the translational start of the corresponding genes. + and - designate positive and negative staining results, respectively.

(B) Quantification of GUS activity in transgenic *L. corniculatus* plants. The data shown represent mean values in units of *p*mol 4-methylumbelliferone (MU) *per* minute *per* mg protein. The error bar represents the standard deviation of the mean. The number of transgenic plants tested is indicated with an n.

A


GUS ACTIVITY (STAINING)
Constructs #
Nodule central tissues
Nodule parenchyma and vascular tissues
Root tissues
Stem and leaf tissues
1 - + - -
2 - - - -
3 - + - -
4 - - - -
5 - - - -

B


nodule
root
n=16
n=18
n=19
n=21
n=31
0
50
100
150
200
250
300
350
GUS activity (pmol MU/min/mg protein)

gus reporter gene was histochemically and quantitatively determined in tissues of transgenic plants 21 days after *Rhizobium* inoculation.

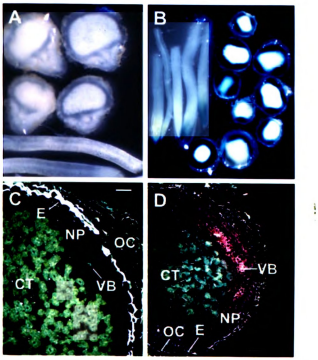
Histochemical staining revealed that *gus* reporter gene expression was visible as a ring-like pattern in cross sections of mature nodules expressing the 5'*SrEnod2-gus*-3'*SrEnod2* construct (Figure 2-3B). No *gus* reporter gene expression could be detected in leaves, stems, or uninfected roots of the transgenic plants (Figure 2-2A). However, weak reporter gene expression could be detected in regions near the root tips of some transgenic plants, but only after *Rhizobium* infection (Figure 2-3B). When the 3'*nos* region was present instead of the *SrEnod2* 3' region, no reporter gene expression could be detected in any tissues of the transgenic plants (Figures 2-2; and 2-3A).

Dark-field microscopy revealed that the expression of the 5'*SrEnod2-gus*-3'*SrEnod2* was localized in the nodule parenchyma and vascular tissues (Figure 2-3D). A low level of GUS activity was also detected in the outer cortex of the nodule. The histochemical staining pattern of the reporter gene expression correlates well with the *in-situ Enod2* mRNA hybridization pattern found in *S. rostrata* nodules (Figure 2-1) and those observed for other legume plants (van de Wiel, 1990a). The only exception to this correlation is that our reporter gene construct was also expressed in the nodule vascular tissue. Dark-field microscopy confirmed that no GUS

LEGEND FOR FIGURE 2-3. Microscopic analysis of the expression of reporter gene constructs in transgenic *L. corniculatus* plants.

(A) Hand-cut sections of transgenic nodules harboring 5'*SrEnod2-gus*-3'*nos* construct (Figure 2-2A, construct 2).
(B) Hand-cut sections of transgenic nodules harboring the 5'*SrEnod2-gus*-3'*SrEnod2* construct (Figure 2A, construct 1). GUS activity (blue color) can be seen in the nodule parenchyma and in regions near the root tip of selected infected roots.
(C) Dark-field microscopy of a 10-μm section of one of the transgenic nodules shown in (A).
(D) Dark-field microscopy of a 10-μm section of one of the transgenic nodules shown in (B). GUS activity (red color) can be seen in the nodule parenchyma, vascular, and outer cortical tissues.

CT, central tissue; E, endodermis; NP, nodule parenchyma; OC, outer cortex; and VB, vascular tissue.


A
B
C
E
NP
OC
CT
VB
D
CT
VB
NP
OC
E

activity was detectable in nodules of transgenic plants harboring the 5'*SrEnod2-gus*-3'*nos* construct (Figure 2-3C).

The histochemical staining analysis revealed limited variations in the expression of the reporter gene. Although 12 out of 17 transgenic plants harboring 5'*SrEnod2-gus*-3'*SrEnod2* (Figure 2-2A, construct 1) had significant reporter gene expression in nodules and 5 plants were inactive, one plant had a high level of reporter gene expression in both nodules and infected roots (data not shown). A variation in reporter gene expression pattern was also observed in one out of 18 transgenic plants harboring the 5'*SrEnod2-gus*-3'*nos* construct, which had a low level of *gus* expression in the nodule vascular tissue (data not shown). It is likely that these variations are due to "position effects" (Odell et al., 1985; Sanders et al., 1987; Benfey et al., 1989), and were therefore excluded from further analysis.

GUS enzymatic activity in nodules and infected roots of transgenic plants was quantified using a fluorometric assay. As shown in Figure 2-2B, GUS activity was detected only in nodules of transgenic plants harboring the 5'*SrEnod2-gus*-3'*SrEnod2* construct. No GUS activity was detected in nodules of transgenic plants harboring the 5'*SrEnod2-gus*-3'*nos* construct, confirming the results of the histochemical staining analysis. These results strongly suggest that the 3' region of the *SrEnod2* gene is required for tissue-specific reporter gene expression in transgenic plants.

In previous studies examining the interaction of DNA-binding proteins (*trans*-acting factors) with the *SrEnod2* locus, a specific protein-DNA interaction with sequences encoding the putative *SrEnod2* leader peptide was identified (A. Goel and F.J. de Bruijn, unpublished observation). Therefore, we also examined the role of the *SrEnod2* putative signal peptide sequence in modulating *gus* reporter gene expression. A 75-bp nucleotide sequence encoding the N-terminal putative signal peptide of 25 amino acids (Dehio and de Bruijn, 1992) was fused to the truncated *SrEnod2* 5' flanking region previously used (Figure 2-2A, constructs 1 and 2). Both in-frame translational and transcriptional fusions to the *gus* coding region were constructed (see Methods). The transcriptional fusions (Figure 2-2A, constructs 3 and 4) displayed a pattern and level of reporter gene expression similar to the original truncated constructs, lacking the leader sequence (Figure 2-2). The putative signal peptide sequence, therefore, does not appear to influence the expression of the reporter gene, when fused to it in a transcriptional configuration. In transgenic plants harboring the in-frame translational fusion (Figure 2-2A, construct 5), however, the *gus* reporter gene was not expressed (Figure 2-2B). Since the reporter gene transcript could be detected in nodules of transgenic plants harboring this gene fusion, albeit at a lower level than that observed with the transcriptional fusion (see below), the lack of GUS activity may be due to poor accumulation of GUS protein or to protein inactivation due to N-linked glycosylation in the

glycosylation in the endoplasmic reticulum during secretion, because of the presence of the putative leader sequence (Farrell and Beachy, 1990).

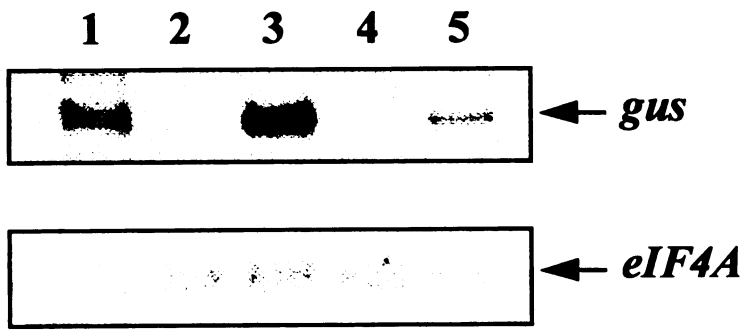
Gus reporter gene activities correlate with steady state *gus* mRNA levels in transgenic nodules

Northern blot analysis was performed to determine whether the *gus* reporter gene activities observed correlate with the accumulation levels of the reporter gene transcript. As shown in Figure 2-4, nodule poly(A)$^+$ RNA isolated from a pool of three independent transgenic plants harboring constructs containing the *SrEnod2* 3' region, strongly hybridized to the riboprobe prepared from the *gus* coding region (Figure 2-4, lanes 1, 3, and 5). The message detected was approximately 2.3 kb in length, as expected (Dehio and de Bruijn, 1992; R. Chen and F. J. de Bruijn, unpublished data). In contrast, nodule poly(A)$^+$ RNA isolated from a pool of three independent transgenic plants harboring constructs containing the 3' *nos* terminator did not hybridize to the *gus* probe (Figure 2-4, lanes 2 and 4). The same blot was stripped and reprobed with an *eIF4A* gene probe (Taylor et al., 1993) to standardize loading.

The signal peptide sequence of the *SrEnod2* gene did not influence the accumulation of the reporter gene transcript when fused in a transcriptional configuration to the *gus* coding region (Figure 2-4, lanes 1 and 3). The *gus*

LEGEND FOR FIGURE 2-4. Northern blot analysis of *gus* reporter gene expression in transgenic nodules harboring the reporter gene constructs 1-5 (Figure 2A).

The lane numbers correspond to the construct numbers. Two micrograms of poly(A)$^+$ RNA were loaded in each lane and hybridized with a ^{32}P-labeled riboprobe derived from the *gus* coding region. The same blot was stripped and rehybridized with a ^{32}P-labeled *eIF4A* probe (Taylor et al., 1993) as a loading control.


1
2
3
4
5
gus
eIF4A

reporter gene transcript also accumulated in nodules harboring the translational gene fusion (Figure 2-2A, construct 5), although the level of *gus* mRNA accumulation was reduced to approximately 50% of that of the transcriptional gene fusion (Figure 2-4, lanes 3 and 5).

These data clearly show that the accumulation of the reporter gene transcript correlates well with the *gus* reporter activity detected in transgenic plants. Additionally, they reveal that the reporter gene transcript accumulates in transgenic plants harboring the constructs containing the *SrEnod2* 3' region, but not the 3' *nos* terminator.

The 3' region of the *SrEnod2* gene contains sequence determinants sufficient for expression in the nodule parenchyma

To further examine the role of the *SrEnod2* 3' region in directing nodule parenchyma-specific expression, additional chimeric reporter gene fusions were constructed containing heterologous promoters, e.g. the leghemoglobin (*Srglb3*) promoter from *S. rostrata* (Szabados et al., 1990; Szczyglowski et al., 1994), the nopaline synthase minimal promoter (-150 bp to +1 bp; Depicker et al., 1982; Ebert et al., 1987), and the CaMV35S minimal promoter (-90 bp to +6 bp; domain A; Franck et al., 1980; Pietrzak et al., 1986; Benfey et al., 1989). The resulting constructs (see Figure 2-5A)

were introduced into *L. corniculatus*, and the expression of the *gus* gene was determined histochemically and quantitatively.

It has previously been shown that a 5'*Srglb3*-*gus*-3'*nos* construct (Figure 2-5A, construct 6) is expressed exclusively in the infected cells of transgenic nodules (Szabados et al., 1990; Szczyglowski et al., 1994; see also Figure 2-6A). However, when the 3' *nos* terminator was replaced by the *SrEnod2* 3' region (Figure 2-5A, construct 7), GUS activity was detected not only in the infected cells of transgenic nodules, but also in the nodule parenchyma cells, the vascular tissue, and the outer cortex (Figures 2-6B and D). Dark-field microscopy revealed that GUS expression directed by the 5'*Srglb3*-*gus*-3'*SrEnod2* construct could also be detected in the central uninfected (interstitial) cells of transgenic nodules (Figure 2-6D), but could not be detected in transgenic stem, leaf, and root tissues (Figure 2-5). This expression pattern suggests an additive effect of the leghemoglobin promoter and the *SrEnod2* 3' region. In addition, the average expression level of the 5'*Srglb3*-*gus*-3'*SrEnod2* construct was consistently higher than the 5'*Srglb3*-*gus*-3'*nos* construct (Figure 2-5B).

The 5'*CaMV35S*(-90/+6)-*gus*-3'*nos* construct (Figure 2-5A, construct 8) did not direct detectable levels of *gus* expression in either nodules or roots of transgenic plants (Figures 2-5, and 2-6E). However, when the 3'*nos* terminator was replaced by the 3' *SrEnod2* region (Figure 2-5A, construct 9), *gus* expression was detected in the nodule parenchyma, vascular

LEGEND FOR FIGURE 2-5. Structure of *gus* fusion constructs and determination of GUS activity in transgenic *L. corniculatus* plants.

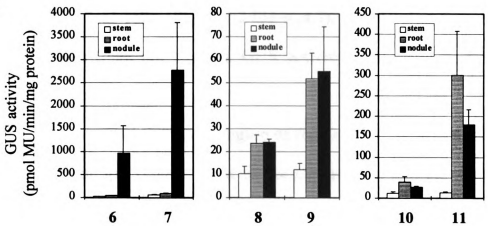
(A) Structure of *gus* fusion constructs. The construct numbers are listed on the left. The open box with angled lines represents the *S. rostrata* leghemoglobin gene promoter (5'*Srglb3*; 1.4 kb). The open box with horizontal lines represents the CaMV35S minimal promoter (CaMV35S; -90 to +6 bp). The open box with vertical lines represents the nopaline synthase minimal promoter (*Pnos*; -150 to +1 bp). For other symbols used see the legend of Figure 2A.

(B) Quantification of GUS activity in transgenic *L. corniculatus* plants. The data represent the mean values. The error bars represent the standard deviation of the mean. The construct numbers are listed at the bottom.

A

CONSTRUCTS #		GUS ACTIVITY (STAINING) Nodule central tissues	Nodule parenchyma and vascular tissues	Nodule outer cortex	Root tissues
6	-1400bp 5'Srglb3 gus 3'nos	+	-	-	-
7	-1400bp 5'Srglb3 gus 3' SrEnod2	++	+	+	-
8	CaMV35S(-90/+6bp) gus 3'nos	-	-	-	-
9	CaMV35S(-90/+6bp) gus 3' SrEnod2	-	+	-	+
10	Pnos(-150/+1bp) gus 3'nos	-	-	-	+
11	Pnos(-150/+1bp) gus 3' SrEnod2	-	+	+	++

B



bundles, and tissues surrounding the vascular bundle connecting the nodule to the root central cylinder (Figures 2-6F and G). *gus* expression was also detected in root vascular tissues, but not in stems or leaves of transgenic plants (data not shown). Fluorometric measurements confirmed this observation (Figure 2-5B).

The 5'*nos*(-150/+1)-*gus*-3'*nos* construct (Figure 2-5A, Construct 10) did not direct detectable levels of *gus* expression in nodules, but was weakly expressed in the root tissues of transgenic plants (Figure 2-5B). Again, when the 3' *nos* terminator was replaced by the 3' *SrEnod2* region (Figure 2-5A, construct 11), *gus* expression could be readily detected in the nodule parenchyma, vascular bundles, and outer cortex, as well as in root vascular tissues and cortical cells (Figures 2-6H and I). No staining was found in stems or leaves (data not shown). GUS activity measurements confirmed these observations (Figure 2-5B).

These data reveal that the 3' region of the *SrEnod2* gene can extend the expression of the *gus* reporter gene to the parenchyma and vascular tissues of transgenic plants, when fused to heterologous promoters, indicating that the *SrEnod2* 3' region contains sequence determinants necessary and sufficient for directing reporter gene expression in the nodule parenchyma and vascular tissues. Moreover, these data reveal that the *SrEnod2* 3' region neither inhibits reporter gene expression in those tissues where the respective 5' (promoter) regions are normally expressed, nor extends

LEGEND FOR FIGURE 2-6. Microscopic analysis of the expression of *gus* fusion constructs in transgenic *L. corniculatus* plants. Staining for GUS activity was carried out overnight, or for 1 hr as indicated.

(A) Hand-cut section of a transgenic nodule harboring the 5'*Srglb*3-*gus*-3'*nos* construct (Figure 2-5A, construct 6). GUS activity (blue color) can be seen in the nodule central tissue (CT). Staining was carried out for 1 hr.

(B) Hand-cut section of a transgenic nodule harboring the 5'*Srglb*3-*gus*-3'*SrEnod2* construct (Figure 2-5A, construct 7). GUS activity (blue color) can be seen in the nodule central tissue, as well as in surrounding tissues. Staining was carried out for 1 hr.

(C) Dark-field microscopy of the nodule section shown in (A). GUS activity (blue color) can be seen in the infected cells of the nodule central tissue.

(D) Dark-field microscopy of the nodule section shown in (B). GUS activity (blue and red colors) can be seen in the nodule central tissue (CT), as well as in the nodule parenchyma (NP), vascular bundles (VB), and outer cortical tissue (OC).

(E) Dark-field microscopy of a transgenic nodule section harboring the 5'CaMV35S(-90/+6)-*gus*-3'*nos* construct (Figure 2-5A, construct 8).

(F) and (G) Dark-field microscopy of a proximal and a distal view of a transgenic nodule section, harboring the 5'CaMV35S(-90/+6)-*gus*-3'*SrEnod2* construct (Figure 2-5A, construct 9). GUS activity can be seen in the nodule parenchyma (NP), vascular tissue bundles (VB) and the root central cylinder (CC). No GUS activity is observed in the root cortex.

(H) and (I) Dark-field microscopy of a proximal and a distal view of a transgenic nodule section harboring the 5'*nos*(-150/+1)-*gus*-3'*SrEnod2* construct (Figure 2-5A, construct 11). GUS activity can be seen in the nodule parenchyma (NP), vascular bundles (VB) and outer cortical tissue (OC), as well as in the root central cylinder (CC) and in the root cortex (RC).

(J) Dark-field microscopy of a transgenic nodule section harboring the CaMV35S (-150/-90)-*5'SrEnod2*(-115/+23)-*gus*-3'*SrEnod2* construct (Figure 2-8, construct 22). GUS activity can be seen in the nodule parenchyma (NP), the nodule vascular bundles (VB) and the nodule central tissue (CT).

(K) and (L) Dark-field microscopy of nodule and root-sections harboring the CaMV35S(-150/-90)-*5'SrEnod2*(-50/+23)-*gus*-3'*SrEnod2* construct (Figure 2-8, construct 23). GUS activity can be seen in the nodule parenchyma (NP), vascular bundles (VB) and central tissue (CT), as well as in the root central cylinder (CC) and cortex (RC). Staining was carried out for 1 hr.

A
VB
NP
CT
B
VB
NP
CT
C
CT
VB
NP
E
OC
D
OC
VB
NP
E
CT
E
CT
NP
VB
F
CT
NP
CC
RC
G
VB
NP
OC
CT
H
CT
NP
CC
RC
J
CT
NP
VB
K
CT
NP
CC
RC
L
CC
RC

reporter gene expression to tissues where the 5' (promoter) regions used are not expressed. Rather, the *SrEnod2* 3' region appears to enhance reporter gene expression in those tissues to which the 5' regions direct expression. It is possible that the same regulatory element in the *SrEnod2* 3' region is responsible for both nodule parenchyma-specific expression and general enhancement of gene expression. Alternatively, separate mechanisms (*cis*-elements) may be responsible for the observed alterations in gene expression.

Delimitation of sequences in the 5' proximal region of the *SrEnod2* gene required for detectable levels of gene expression

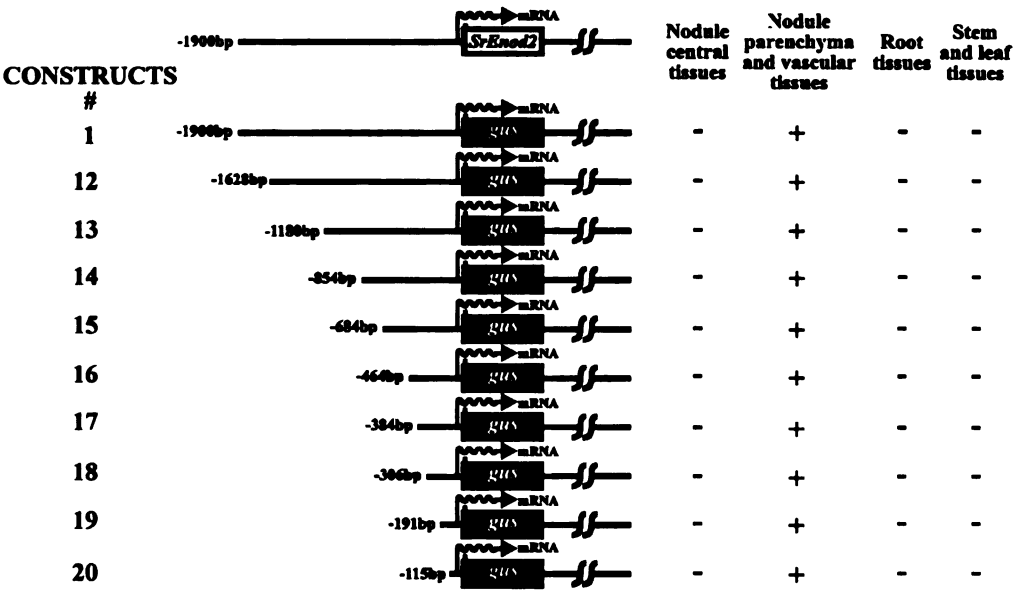
A series of 5'→3' unidirectional deletions in the 5' *SrEnod2* region was constructed and fused to the *gus* coding region, as well as to the 3' region of the *SrEnod2* gene (Figure 2-7A). The resulting constructs were introduced into *L. corniculatus* plants and reporter gene expression was analyzed histochemically and quantitatively. GUS activity measurement revealed that constructs with deletions up to position -191, relative to the transcriptional start site, directed reporter gene expression in the same fashion as the full 5'*SrEnod2-gus*-3'*SrEnod2* construct (Figure 2-2A, construct 1), namely to the nodule parenchyma and vascular tissues (Figure 2-7A and B). Only the level of GUS enzymatic activity varied slightly (Figure 2-7B). These data

LEGEND FOR FIGURE 2-7. Structure of *SrEnod2* 5' deletion reporter gene constructs and GUS activity in transgenic *L. corniculatus* plants.

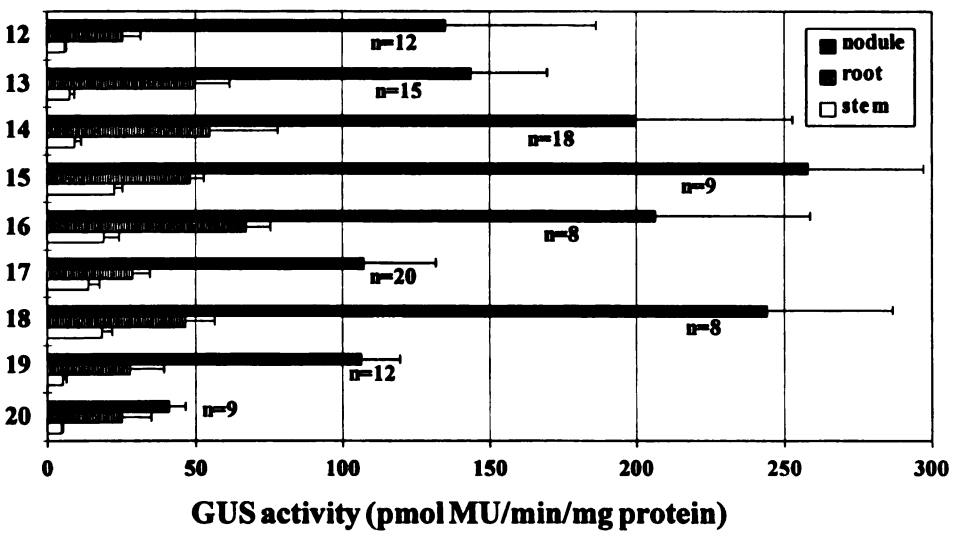
(A) Structure of *gus* fusion constructs. The construct numbers are listed on the left. The base pair (bp) numbers indicate the end point of the 5' deletion relative to the start of transcription. The vertical bars attached to the boxes represent the translational start of the corresponding genes. The interrupted lines downstream of the *gus* reporter gene represent the 2.4-kb 3' flanking sequences of the *SrEnod2* gene. + and - represent positive and negative GUS staining activity, respectively. For other designations, see the legend of Figure 2A.

(B) Quantification of GUS activity in transgenic *L. corniculatus* plants. The data represent the mean values. The error bar represents the standard deviation of the mean. The number of transgenic plants tested is indicated with an n.

A


GUS ACTIVITY (STAINING)
Nodule central tissues
Nodule parenchyma and vascular tissues
Root tissues
Stem and leaf tissues
CONSTRUCTS
#
-1900bp
SrEnod2
mRNA
gus
1
-1900bp
- + - -
12
-1628bp
- + - -
13
-1180bp
- + - -
14
-854bp
- + - -
15
-684bp
- + - -
16
-464bp
- + - -
17
-384bp
- + - -
18
-306bp
- + - -
19
-191bp
- + - -
20
-115bp
- + - -

B

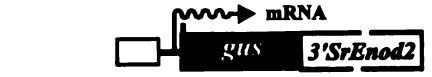

12
13
14
15
16
17
18
19
20
n=12
n=15
n=18
n=9
n=8
n=20
n=8
n=12
n=9
nodule
root
stem
0
50
100
150
200
250
300
GUS activity (pmol MU/min/mg protein)

suggest that sequences up to position -191 are not required for nodule parenchyma-specific reporter gene expression. A deletion down to position -115 (Figure 2-7A, construct 20) was found to lead to a significant reduction of reporter gene expression in nodules (Figure 2-7B), although a limited degree of nodule enhanced expression was maintained. Histochemical GUS staining revealed that this construct retained *gus* gene expression in the nodule parenchyma and vascular tissues (Figure 2-7A; data not shown). Therefore, we suggest that the sequences down to position -115 may not be absolutely necessary for nodule parenchyma-specific expression.

A construct with a deletion up to position -50 in the 5' *SrEnod2* region was found to be inactive in transgenic plants (Figure 2-8). To test whether the lack of reporter gene expression was due to a deletion of tissue-specific *cis*-element(s) or to a deletion of proximal promoter element(s), the transcriptional enhancer from the CaMV35S promoter (-150 to -90 bp, part of domain B; Benfey et al., 1989) was fused in *cis* to the -50 bp fragment (Figure 2-8, construct 23). GUS activity measurement in transgenic plants harboring this construct revealed that the reporter gene was strongly expressed in nodule, root, and stem tissues (Figure 2-8). Within nodules, the reporter gene was highly expressed in the nodule parenchyma and vascular tissues, and to a lower level in the infected tissue (Figure 2-6K). Within roots, the *gus* gene was expressed in the central cylinder and cortical

LEGEND FOR FIGURE 2-8. Structure of *SrEnod2* 5' deletion reporter gene constructs, enhancer fusions and GUS activity in transgenic *L. corniculatus* plants.

The construct numbers are listed on the left. The base pair (bp) numbers indicate the end point of the 5' deletion, relative to the start of transcription. The interrupted open box represents the 2.4-kb 3' flanking region of the *SrEnod2* gene. The open box represents the transcriptional enhancer of the CaMV35S promoter (-150 to -90 bp). For other designations, see the legend of Figure 2A.

CONSTRUCTS #		GUS ACTIVITY (STAINING)			
		Nodule central tissues	Nodule parenchyma and vascular tissues	Root tissues	Stem tissues
20		-	+	-	-
21		-	-	-	-
22		+	+	+	+
23		++	++	++	++

tissues (Figure 2-6L). When the same transcriptional enhancer was fused to the -115 bp deletion fragment of the 5' *SrEnod2* gene (Figure 2-8, construct 22), an identical GUS activity staining pattern was observed as with construct 23 (Figures 2-6J, and 2-8), although the GUS activity was much lower than plants harboring construct 23 (Figure 2-8; and data not shown). These data suggest that sequences between positions -115 and -50 in the 5' *SrEnod2* region are necessary for reporter gene expression. However, they may not contain any tissue-specific *cis*-elements.

The 3' untranslated region (3' UTR) of the *SrEnod2* gene constitutes the minimum determinant for nodule parenchyma-specific gene expression

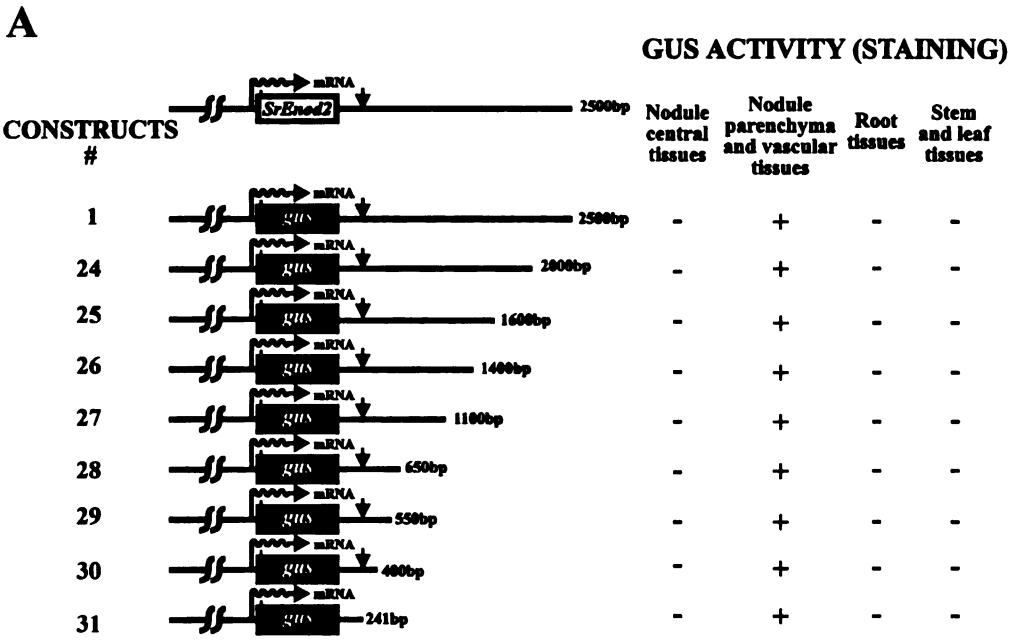
To delineate the sequence determinants in the 3' region of the *SrEnod2* gene responsible for nodule parenchyma-specific expression, a series of 3'→5' unidirectional deletions in the 3' region of the *SrEnod2* gene were constructed (Figure 2-9A). The 3' deletion derivatives were fused to the *gus* coding region, flanked by the full-length 5' *SrEnod2* region. The resulting constructs were introduced into *L. corniculatus,* and reporter gene expression was determined. As shown in Figure 2-9, deletions in the 3' region up to position +400 bp relative to the stop codon, did not affect tissue-specific expression of the reporter gene (Figure 2-9, construct 30). The level of gene expression was greatly reduced when the 3' flanking

LEGEND FOR FIGURE 2-9. Structure of *SrEnod2* 3' deletion reporter gene constructs and determination of GUS activity in transgenic *L. corniculatus* plants.

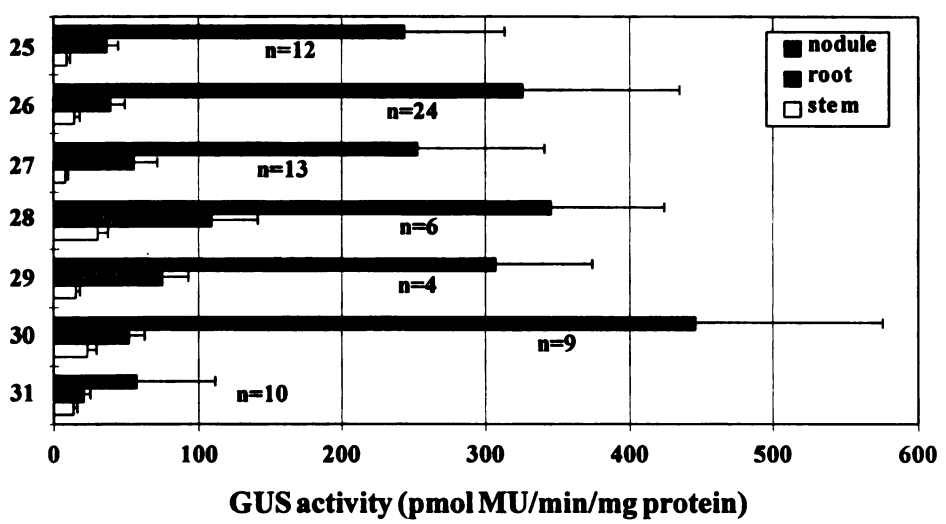
(A) Structure of *gus* fusion constructs. The construct numbers are listed to the left. The base pair (bp) numbers indicate the end point of the 3' deletion, relative to the stop codon of the *SrEnod2* gene. The vertical bars attached to the boxes represent the translational start of the corresponding genes. The vertical arrow indicates the position of the polyadenylation site of the *SrEnod2* gene. The interrupted lines at the 5' end represent the 1.9-kb 5' *SrEnod2* region.

(B) Quantification of GUS activity in transgenic *L. corniculatus* plants. The data represent the mean values. The error bar represents the standard deviation of the mean. The number of transgenic plants tested is indicated with an n.

A


GUS ACTIVITY (STAINING)
CONSTRUCTS #
Nodule central tissues
Nodule parenchyma and vascular tissues
Root tissues
Stem and leaf tissues
mRNA
SrEnod2
2500bp
1
gus
2500bp
- + - -
24
2000bp
- + - -
25
1600bp
- + - -
26
1400bp
- + - -
27
1100bp
- + - -
28
650bp
- + - -
29
550bp
- + - -
30
400bp
- + - -
31
241bp
- + - -

B


25
n=12
26
n=24
27
n=13
28
n=6
29
n=4
30
n=9
31
n=10
nodule
root
stem
0
100
200
300
400
500
600
GUS activity (pmol MU/min/mg protein)

sequences were further limited to position +241 bp, although the nodule parenchyma-specific expression pattern appeared to be maintained (Figure 2-9, construct 31; data not shown). Therefore, the 241-bp fragment immediately downstream of the stop codon of the *SrEnod2* gene, containing most of the *SrEnod2* 3' untranslated sequence (Dehio and de Bruijn, 1992; R. Chen and F. J. de Bruijn, unpublished data), appears to be sufficient to direct gene expression to the nodule parenchyma and vascular tissues.

DISCUSSION

The 3' UTR of *SrEnod2* confers nodule parenchyma-specific expression

The *Enod2* genes of several legumes have been shown to be expressed in the nodule parenchyma cell layer (van de Wiel et al., 1990a, 1990b; Allen et al., 1991; Figure 2-1). Here we show that in the case of the tropical legume *S. rostrata*, the (primary) determinant of this cell-specific expression pattern is located in the 3' UTR of the *SrEnod2* gene. The following lines of evidence support this conclusion: a) Chimeric *gus* reporter constructs flanked by both 5' and 3' regions of the *SrEnod2* gene are expressed in the parenchyma of *transgenic L. corniculatus* nodules, while constructs containing a heterologous 3' region (e.g. 3' *nos*) are not (Figures 2-2, and 2-3); b) The presence of the *SrEnod2* 3' region is necessary for the

accumulation of the *gus* mRNA in transgenic nodules (Figure 2-4); and c) The addition of the *SrEnod2* 3' region to chimeric reporter gene constructs containing heterologous promoters (e.g., *Srglb3* promoter, CaMV35S minimal promoter, nopaline synthase minimal promoter) extends the expression pattern normally observed with the promoters to the nodule parenchyma (Figure 2-5).

One interesting observation during our analysis of *gus* reporter gene expression was that all the *gus* constructs containing the *SrEnod2* 3' region directed *gus* expression also to the vascular bundles of transgenic *L. corniculatus* nodules. This observation contrasts with our *in-situ* hybridization results (Figure 2-1), which did not reveal *SrEnod2* mRNA accumulation in the vascular bundles. It also contrasts with previous results on soybean *Enod2* gene/promoter expression, which did not reveal reporter gene expression in the vascular tissues either (Lauridsen et al., 1993). This observation suggests that the *gus* expression assay is more sensitive than *in-situ* hybridization, because of the stability of GUS protein (Jefferson et al., 1987), and that the expression pattern of the *S. rostrata Enod2* gene differs in this respect from the soybean homologue. The latter is quite plausible, since the *S. rostrata Enod2* gene is inducible by cytokinin (Dehio and de Bruijn, 1992; Silver et al., 1996), whereas the soybean *Enod2* gene is not. Moreover, in the case of the soybean *Enod2* gene, nodule parenchyma-specific expression has been reported to be controlled by elements far

upstream in the 5' region of the gene (Lauridsen et al., 1993; also see below).

Another interesting observation was made with the chimeric 5'*Srglb*3-*gus*-3'*SrEnod2* construct. This construct was found to direct *gus* expression not only in the nodule parenchyma, vascular bundles, and infected cells of the central tissue, suggesting an additive effect of cell specificity mediated by the *SrEnod2* 3' region and the *Srglb*3 promoter (Szczyglowski et al., 1994), but also in the uninfected cells of the central tissue (Figure 2-6D). This unexpected expression pattern may be due to an unusual interaction of the *cis*-acting elements in these 5' and 3' regions. Alternatively, since the results shown in Figure 2-6 suggest that the *SrEnod2* 3' region appears to confer both (a) tissue specific, as well as (an) enhancer function(s), it may reflect an enhancement of *Srglb*3 promoter activity in the uninfected cells to a previously undetectable level (Szczyglowski et al., 1994).

Our *SrEnod2* 3' deletion analysis revealed that the DNA sequences downstream of position +400 bp, relative to the *SrEnod2* stop codon, can be omitted without affecting the expression pattern of chimeric reporter gene constructs (Figure 2-9). In fact, even a deletion construct lacking DNA sequences 3' to position +241 directs expression to the nodule parenchyma, while expression in roots and stems/leaves is virtually non-detectable (Figure 2-9). This suggests that sequences between the *SrEnod2* stop codon and position +241, upstream of the poly A addition site (around position

+258, Dehio and de Bruijn, 1992; R. Chen and F. J. de Bruijn, unpublished result), are capable of directing cell-specific expression in transgenic nodules, albeit at a low level. It is possible that the enhancer-like activity of the *SrEnod2* 3' region is mediated by sequences located between positions +241 and +400, whereas the cell-specificity determinant(s) lie(s) in the 3' UTR. Further analysis will be required to support this hypothesis.

DNA elements in the 3' region of plant genes have previously been implicated in regulation of gene expression. Examples include the potato proteinase inhibitor II (*PI-II*) gene (Thornburg et al., 1987; An et al., 1989), the petunia ribulose bisphosphate carboxylase small subunit gene (Dean et al., 1989), the oilseed rape *AX92* gene (Dietrich et al., 1992), the Arabidopsis *GLABROUS1* (*GL1*) (Larkin et al., 1993), and the potato sucrose synthase (*Sus4; Sus3*) gene (Fu et al., 1995a and b). Multiple examples have also been reported in mammalian systems. Examples include the chicken adult *β-globin* gene (Choi et al., 1986), the chicken histone *H5* gene (Trainor et al., 1987), the human A_γ globin gene (Bodine and Ley, 1987), the mammalian ribonucleotide reductase R1 gene (Chen et al., 1993), and the human transferrin receptor (*TfR*) gene (Owen and Kuhn 1987; Binder et al., 1994). In these cases, the 3' sequences act either as transcriptional enhancers or sequence determinants modulating gene expression post-transcriptionally. *Cis*-element(s) acting as transcriptional enhancers are generally located downstream of poly(A) addition site(s) and

often act as tissue-specific and/or developmental regulatory elements (e.g., Arabidopsis *GL1* gene; chicken adult β-*globin* gene; chicken histone *H5* gene; human A_γ globin gene). In those cases where post-transcriptional regulation is involved, the 3' sequences modulate mRNA stability (petunia *rbcs* genes; human *TfR* gene), or efficacy of mRNA 3' end formation and processing (potato *PI-II* gene). Additionally, different 3' end regions have also been found to influence the level of gene expression in plant cells (Ingelbrecht et al., 1989).

However, our finding that (a) *cis*-element(s) responsible for cell-specific expression is located within the 3' UTR appears to be a unique example amongst plant nuclear genes. The mechanism by which the *SrEnod2* 3' UTR mediates nodule parenchyma-specific expression remains to be elucidated. In a separate but related study, we have shown that cytokinin-mediated enhancement of *SrEnod2* gene expression in *S. rostrata* roots, in the absence of rhizobial infection, appears to occur at a post-transcriptional level (Silver et al., 1996). This aspect of *SrEnod2* regulation also involves the 3' UTR of the gene (D. Silver, R. Chen, and F.J. de Brujin, unpublished observations). Whether nodule parenchyma-specific expression and cytokinin up-regulation of the *SrEnod2* gene share common regulatory elements is currently unknown; however, our data favor the hypothesis that both processes involve post-transcriptional regulation. Recently, cell-specific mRNA stability elements have been identified in the 3' UTR of the

α2-globin gene (Weiss and Liebhaber, 1995). It is possible that the *SrEnod2* 3' UTR directs nodule parenchyma-specific expression in a similar way as the 3' UTR of the *α2-globin* gene. Alternatively, the *SrEnod2* 3' UTR may act as a tissue-specific transcriptional enhancer. Experiments are in progress to address these possibilities and to further delimit the responsible *cis*-elements.

The 5' region of *SrEnod2* does not appear to harbor significant tissue-specific *cis*-elements

Our deletion analysis of the *SrEnod2* 5' region revealed that sequences upstream of position -191, relative to the transcriptional start site, are not required for nodule parenchyma-specific expression. A deletion up to the -50 bp position abolishes reporter gene expression in nodules, but fusing a transcriptional enhancer (CaMV35S -150 bp to -90 bp; see Szabados et al., 1990) to the -50 bp fragment restores reporter gene expression to the nodule parenchyma/vascular tissue, to stem and root tissues, and to a limited extent to the nodule central tissue (Figure 2-6K). This pattern of reporter gene expression was found to be similar to the pattern directed by a fusion of the same transcriptional enhancer to the -115 bp fragment of the *SrEnod2* 5' region (Figure 2-6J). These data suggest that sequences between positions -115 and -50 are required for a significant level of reporter gene expression

in nodules. However, these sequences are not likely to contain (a) nodule tissue-specific *cis*-element(s).

The latter conclusion is very intriguing in light of the fact that, in the case of the soybean *Enod2* (B) gene, Lauridsen et al. (1993), have reported that the 3-kb promoter region directed reporter gene expression to the parenchyma of *L. corniculatus* and *Trifolium repens* transgenic hairy root nodules. The 3' *nos* terminator was used in their study. Two critical tissue-specific *cis*-elements were identified in regions located between positions -1792 and -1582, and between -380 and -53, relative to the transcriptional start site. When the upstream tissue-specific *cis*-element was fused to the promoter region of a leghemoglobin gene (*lb*), the resulting gene fusion was found to direct a low level of reporter gene expression in a scattered pattern in the nodule central area. These data appear to be contradictory to the data presented in this paper, and/or suggest further that there are profound differences in the mechanisms regulating *SrEnod2* and the soybean *Enod2(B)* gene expression (also see discussion above). Further support for this idea is the finding that DNA sequences in the promoter regions of the *SrEnod2* and the soybean *Enod2*(B) genes share remarkably little homology (de Bruijn et al., 1994), in contrast to the late nodulin leghemoglobin (*lb*) promoters, which are highly similar (de Bruijn et al., 1990; de Bruijn and Schell, 1992; Szabados et al., 1990; Szczyglowski et al., 1994; Stougaard et al., 1990; Ramlov et al., 1993). Perhaps this discrepancy can be explained

once the nucleotide sequences of the *cis*-element(s) responsible for the nodule parenchyma-specific gene expression are identified and directly compared.

In any case, the apparent involvement of the 3' region of the *SrEnod2* gene, rather than its promoter (5') region, in nodule parenchyma-specific expression is unique in a variety of ways and opens up a whole new area of investigations on the mechanisms of plant gene expression in response to rhizobial infection.

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CHAPTER 3

IDENTIFICATION OF *CIS*-ELEMENTS WITHIN THE 3' UNTRANSLATED REGION OF THE *SrENOD2* GENE AND INTERACTING PROTEINS

ABSTRACT

Unlike some other plant nuclear genes, whose transcripts are polyadenylated at multiple sites yielding highly heterogeneous 3'-ends, *SrEnod2* transcripts terminate at three consecutive nucleotide positions. Previously, we have shown that the 3' UTR of the *SrEnod2* gene contains *cis*-elements responsible for nodule parenchyma-specific expression. Using deletions and truncations within the 3' UTR that are fused to the *gus* reporter gene in transgenic plants, we have delineated sequence motifs within the *SrEnod2* 3' UTR, deletion of which affects tissue-specific expression. Using a protein-RNA UV-cross linking approach, we have identified several nodule-specific proteins that interact with the RNA transcribed from the *SrEnod2* 3' UTR. Competition experiments have revealed that sequences between positions +1 and +163 nt downstream of the stop codon are efficient to compete out the observed protein-RNA interactions, suggesting that the *cis*-acting elements responsible for nodule parenchyma-specific expression co-localize with specific protein binding sequences.

INTRODUCTION

The *S. rostrata SrEnod2* gene, encoding a putative cell wall protein (Dehio and de Bruijn, 1992), and is expressed in the nodule parenchyma during early stages of both stem and root nodule development (Chen et al., 1996; Chapter 2), as well as in roots after cytokinin treatment (Dehio and de Bruijn, 1992; Silver et al., 1996). By examining *gus* reporter gene expression in transgenic *L. corniculatus* plants, *cis*-elements responsible for the nodule parenchyma-specific expression have been delimited to the 3' untranslated region (3' UTR) of the *SrEnod2* gene (Chen et al., 1996). The same 3' UTR appears to confer cytokinin enhancement of the *gus* reporter gene expression in transgenic Arabidopsis (D. Silver, R. Chen, and F.J. de Bruijn, unpublished data). The presence of the *SrEnod2* 3' UTR is required for accumulation of the *gus* reporter transcript in nodules of transgenic plants, indicating that a post-transcriptional mechanism may be responsible for the observed nodule parenchyma-specific expression.

Cytokinin up-regulation of *SrEnod2* gene expression has been further characterized using nuclear run-off experiments and application of transcriptional and translational inhibitors. It has been shown that cytokinin up-regulation takes place in the cytoplasm, and occurs at a post-transcriptional level (Silver et al., 1996). This process requires concurrent protein synthesis, and appears to involve protein phosphorylation as well

(Silver et al., 1996). These data suggest that *trans*-acting protein factor(s) may be involved in both tissue-specific and cytokinin induced expression of the *SrEnod2* gene by interacting with RNA sequences encoded by the *SrEnod2* 3' UTR.

To test this hypothesis, we carried out RNA-protein UV-cross linking experiments which identified nodule-specific proteins that interact with RNA sequences encoded by the *SrEnod2* 3' UTR. Additionally, the effect of various deletions in and truncations of the 3' UTR of the *SrEnod2* gene was examined in RNA-protein competition experiments, as well as in transgenic *L. corniculatus* plants. These analyses revealed that the *cis*-acting elements required for nodule parenchyma-specific expression co-localize with protein binding sites in the RNA sequences derived from the 3' UTR of the *SrEnod2* gene.

METHODS

Plant growth and nodulation

Sesbania rostrata seeds were surface-sterilized and germinated on petri dishes in the dark at 28^{o}C for one day, as described by Pawlowski et al., (1991). Seedlings were grown for three weeks in autoclaved soil mix (MetroMix:sand; 2:1) in growth chambers with an 18-hr light (28^{o}C) and

6-hr dark (22°C) cycle. Plants were inoculated with a two-day-old culture of *Azorhizobium caulinodans* ORS571 (Dreyfus and Dommergues, 1981) on the stems for stem nodulation. Stem tissues and ten-day-old stem nodules were collected and processed (see below).

Lotus corniculatus transgenic plants were grown for one week in autoclaved soil mix (Vermiculite:sand:MetroMix; 10:10:1) in growth chambers with a 16-hr light (24°C) and 8-hr dark (18°C) cycle. Plants were inoculated with a three-day-old culture of *Rhizobium loti* NZP2037 (Pankhurst, 1983) and grown for 21 days. Plant tissues (nodules, infected roots, and leaves plus stems) were collected and assayed for GUS activity (see below).

End-labeling and S1 nuclease protection assay

The 2.4 Kb SacI-EcoRI fragment of the *SrEnod2* 3' region was previously generated using a site-directed mutagenesis approach (see Methods of Chapter 2). The resulting plasmid was linearized by SacI digestion and the resulting fragment was end-labeled by T4 DNA polymerase, and Klenow in the presence of dCTP, dGTP, dTTP and α^{32}P-dATP. The reaction was controlled in such a way that about 30 nucleotides at each end were labeled (Maniatis et al., 1989). The labeled fragment was digested with HindIII, and the 2 kb SacI end-labeled fragment was purified and used as the S1 probe.

S1 nuclease protection analysis was performed according to Binder et al. (1994). Total RNA was isolated from ten-day-old stem nodules and ethanol precipitated together with $1x10^5$ cpm ($2x10^6$ cpm/pmol) of the prepared S1 probe. Samples were resuspended in 40 µl of 80 % deionized formamide, 0.4 M NaCl, 0.04 M PIPES (pH 6.4) and 0.005 M EDTA, heated at 85°C for 10 min and annealed at 30°C overnight. Samples were diluted with 9 volumes of 0.4 M NaCl, 0.03 M sodium acetate (pH 4.8), 0.001 M $ZnCl_2$, 10 µg/ml of sonicated and denatured salmon sperm DNA and S1 nuclease (1000 units/ml; BRL), and incubated at 37°C for 1 hr. The reaction was terminated by adding of EDTA to a final concentration of 0.015 M, extracted with phenol-chloroform and ethanol precipitated. Alkaline hydrolysis was performed by dissolving dried samples in 100 µl of 0.2 N NaOH and incubation at 42°C for 1 hr. Samples were neutralized with 20 µl of 1 M Tris-HCl (pH 7.4) and 0.2 N HCl, and ethanol precipitated with 10 µ g of carrier yeast tRNA. Samples were washed in 70% ethanol and air dried. Samples were resuspended in 4 µl of loading dye, loaded on 6% polyacrylamide sequencing gels and autoradiographed.

Mapping of 3'-ends of *SrEnod2* mRNA by 3' RACE

Mapping of the 3'-ends was carried out by the 3' RACE (random amplification of cDNA ends) method described by Frohman et al. (1988)

and Frohman (1990). Total RNA was reverse transcribed in 20 µl solution containing 50 mM Tris-HCl (pH 8.15), 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 1 mM dNTP, 0.5 µg of dT_{17}-adapter primer (5'GACTCGAGTCGAC ATCGAT$_{17}$3'), 10 units of RNasin, 10 units of AMV reverse transcriptase, at 42^{o}C for 1 hr. An aliquot of this reaction was used to PCR amplify the 3' ends of the *SrEnod2* transcripts. The PCR reaction was carried out in a solution, containing 70 mM Tris-HCl (pH 8), 7 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 10% DMSO, 1.5 mM dNTPs, 25 pmol adapter primer (5'GACT CGAGTCGACATCG3'), 25 pmol gene-specific primer (5'GAGCTCCAAC CACTACC3'), 1 µl of reverse transcription reaction mixture and 2.5 units of Taq polymerase.

Construction of chimeric reporter gene fusion

The 1.9-kb EcoRI-BamHI fragment, which contains the entire 5' untranslated upstream region of the *SrEnod2* gene (see Methods of Chapter 2), was inserted in front of the *gus* coding region carried on plasmid pBI101.1 (Clontech Laboratories, Palo Alto, CA) to generate the pBI101.1-5'*SrEnod2* vector. The EcoRI end of the fragment was filled in by Klenow before cloning. The 400 bp and the 241 bp deletions in the *SrEnod2* 3' region were generated previously (see Methods of Chapter 2). To generate construct 32, the 400 bp deletion fragment (SacI-XhoI) was digested with

NheI. The resulting 163 bp-long SacI-NheI fragment was inserted into the SacI-XbaI sites of pBluescript KS- vector (Stratagene, La Jolla, CA). A SacI-EcoRI fragment was excised from the resulting plasmid and inserted into the SacI-EcoRI sites of the pBI101.1-5'*SrEnod2* vector. To generate construct 33, the 400 bp deletion fragment was digested with MboI. The resulting 360 bp MboI-XhoI fragment was inserted into the BamHI-XhoI sites of pBS KS- vector. The resulting plasmid was digested with BstXI and SpeI, Klenow filled-in and religated to remove the polylinker sequence between the SacI and BamHI sites. A SacI-XhoI fragment was then excised and inserted into the SacI-XhoI sites of pBI101.1-5'*SrEnod2* vector. To generate construct 34, the 400 bp fragment was digested with NheI, and the resulting 237 bp NheI-XhoI fragment was inserted into the XbaI-XhoI sites of pBS KS- vector. A SacI-XhoI fragment was excised from the resulting plasmid and inserted into the SacI-XhoI sites of the pBI101.1-5'*SrEnod2* vector. To generate construct 35, a 40 bp SacI-MboI subfragment of the 400 bp fragment was first inserted into the SacI-BamHI sites of pBS KS- vector, a 237 bp NheI (blunt-ended by Klenow fill-in)-XhoI subfragment was subsequently inserted at the SmaI-XhoI sites of the resulting plasmid. A SacI-XhoI fragment was then excised and inserted into the SacI-XhoI sites of the pBI101.1-5'*SrEnod2* vector.

Regeneration of Transgenic *Lotus corniculatus* Plants

Transgenic *Lotus corniculatus* cv Rodeo plants were generated according Szabados et al. (1990). Binary vectors were conjugally transferred into the *Agrobacterium rhizogenes* strain A4 (Tempe and Casse-Delbart, 1989). Regenerated plants were transferred to growth chambers (Conviron, Asheville, NC) and nodulated as described (Szabados et al., 1990).

Histochemical Staining of GUS Enzymatic Activity

GUS enzymatic activity was histochemically analyzed according to the procedure described by Jefferson (1987) with the following modifications. Hand-cut nodule sections were incubated at 37^{o}C in a solution containing 50 mM Na_2PO_4 and $NaHPO_4$, pH 7, 1 mM ferri- ferro-cyanide and 20% methanol, and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide; Gold Biotechnology Inc., St. Louis, MO). Stained sections were cleared through an ethanol and ethylene series and analyzed under a dissection microscope (Wild MPS52, Heerburgg, Switzerland).

RNA-protein UV-cross linking experiments

Cellular protein extracts were prepared as following. Plant materials were grinded to a fine powder in liquid nitrogen and resuspended in an equal volume of 2x NP-40 extraction buffer (20 mM HEPES, pH 7.9; 80 mM KCl; 6 mM $MgCl_2$; 2 mM DTT; 10 % glycerol; 0.4 % NP-40; 16 μg/ml Aprotinin; 4 μg/ml Leupeptin; and 1 mM PMSF). After mixing, an equal volume of 1x NP-40 extraction buffer was added. The mixture was centrifuged at 14,000 rpm for 2 min. The supernatant was collected, and protein concentrations were determined according to Bradford (1976). Crude extracts were aliquotted and stored at -70^{o}C until use.

Plasmid pBS KS- DNA, containing the 241 bp 3' UTR fragment was linearized at the XhoI or SacI sites and used as template to produce sense or antisense RNA in *in vitro* transcription reactions using T7 or T3 RNA polymerase, respectively, according to the manufacturer's instructions. Labeled RNA transcripts were produced by adding α^{32}P-UTP (800 Ci/mmol) in the reaction, and the resulting transcripts had a specific activity of approximately 3x 10^7 cpm/μg. Cold RNAs were prepared essentially the same way except that cold UTP was used in the reactions. Poly(A), poly(C), poly(G), and poly(U) (Sigma, St. Louis, MO) were resuspended in 1x binding buffer and stored at -70^{o}C until use.

Cellular protein extracts (10 μg) were incubated at room temperature for 30 min with 125,000 cpm ^{32}P-labeled RNA in 1x buffer containing 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 1 mM dithiothreitol, and 5 % glycerol. The volume of each reaction was 20 μl. RNase-T1 (20 units; Boehringer Mannheim, Mannheim, Germany) was added to cleave unbound RNA, and Heperan sulfate (0.1 mg; Sigma) was added to reduce nonspecific binding. The reaction mixtures were UV-cross linked using 254 nm UV radiation (250 mJ/cm^2) with a Stratagene UV cross-linking apparatus (Stratagene, La Jolla, CA) and then separated by electrophoresis on 10 % SDS-polyacrylamide gels under reducing conditions. Gels were dried and exposed to x-ray films at -70^{o}C.

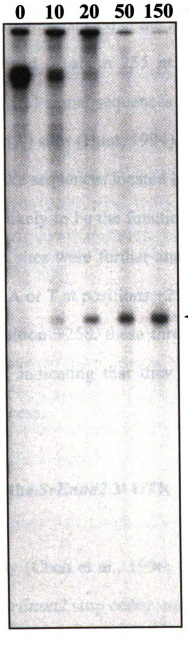
RESULTS

Polyadenylation sites of the *SrEnod2* transcript

Sequence analysis of *SrEnod2* cDNA clones revealed that the *SrEnod2* transcript was polyadenylated at the T residue at position +258 nt downstream of the TAA stop codon (Strittmatter, et al., 1989; Dehio and de Bruijn, 1992). Since multiple AATAAA-like sequences were present in the *SrEnod2* 3' UTR (Figure 3-2), we tested whether multiple polyadenylation events occurred using both S1 nuclease protection and 3' RACE (rapid

LEGEND FOR FIGURE 3-1. S1 nuclease protection assay.

Total RNA of 10-day-old stem nodules of *S. rostrata* was mixed at the concentration indicated on top of each lane with 1x 10^5 cpm S1 probe prepared from the *SrEnod2* 3' region. A single band of 255 nt was protected after S1 nuclease digestion. Bands of higher molecular weights were derived from the double-stranded S1 probe.


0 10 20 50 150 ug RNA
255 nt

amplification of cDNA ends; Frohman et al., 1988). For S1 nuclease protection assays, total RNA isolated from 10 day-old stem nodules of *S. rostrata* plants, hybridized with a 2 kb SacI-HindIII fragment of the *SrEnod2* 3' flanking region labeled at the SacI site. As shown in Figure 3-1, a single protected band of about 255 nt was detected after S1 nuclease digestion. Since poly(A) signal sequences are normally located 10 to 30 nt upstream of the poly(A) sites (Hunt, 1994), a single protected band suggests that the AATAAA-like sequences located between 20 and 30 nt upstream of the poly(A) site are likely to be the functional poly(A) signals (Figure 3-2). The polyadenylation sites were further analyzed using 3' RACE and found to correspond to the A or T at positions +256 and +257. Together with the T residue found at position +258, these three polyadenylation sites are very close to each other, indicating that they may be generated by the same polyadenylation process.

Sequence motifs in the *SrEnod2* 3' UTR

As shown previously (Chen et al., 1996; Chapter 2), the 241 bp fragment downstream of the *SrEnod2* stop codon contains the minimal *cis*-element(s) responsible for nodule parenchyma-specific expression. To delineate sequence motifs within the *SrEnod2* 3' UTR that are important for nodule parenchyma-specific expression, motif deletions and truncations within the

```
CCGGGATACAATCCACCACCATATGGCCACTATCCACCGTCCAAAAAAAATTAA
P  G  Y  N  P  P  P  Y  G  H  Y  P  P  S  K  K  N  *

CAACCACTACCACTACACGTGGCATGCATATTTTGGTGATCTAATAAATGTCAA     54

GATTAGTTGTTTGTCATAGTAAAGTTTTTGTTTTCTCCCTCCTTCCCTTTAATT    108

AATTACCACTTTTAAGATGCATGTGAACTAGCTAGCTACAATGTTCTCCTTCAA    162

AATAAGGGCTCTATGCATGCCCCTCTCTCTGTAACCTTTGCTTTGCATTTCCCT    216

                                        ↓↓↓
TTATCTTGTAATATCAATCAATAAGTACTTTCTCGTTTTATTATCTATGCCTAC    270

CATACAATTACATTCTTTATCATACTTACAAATTAATGATAAGCTAAACGGAAA    324

ATCTAAACATACATAACACACAGTAGATACAATAATTGTAACACTGTTATGTTA    378

GTACCAAAAAAAAAAGAGAGAT
```

FIGURE 3-2. Sequence of the 3' region of the *SrEnod2* gene. A short stretch of C-terminal amino acid sequences is shown. * indicates the stop codon. The underlined nucleotide sequences represent AATAAA-like sequences. Nucleotides in bold represent sequences recognized by the MboI, NheI, and RsaI enzymes, from the 5' to 3'direction, respectively. Arrows indicate the polyadenylation sites identified by using 3' RACE and cDNA sequencing.

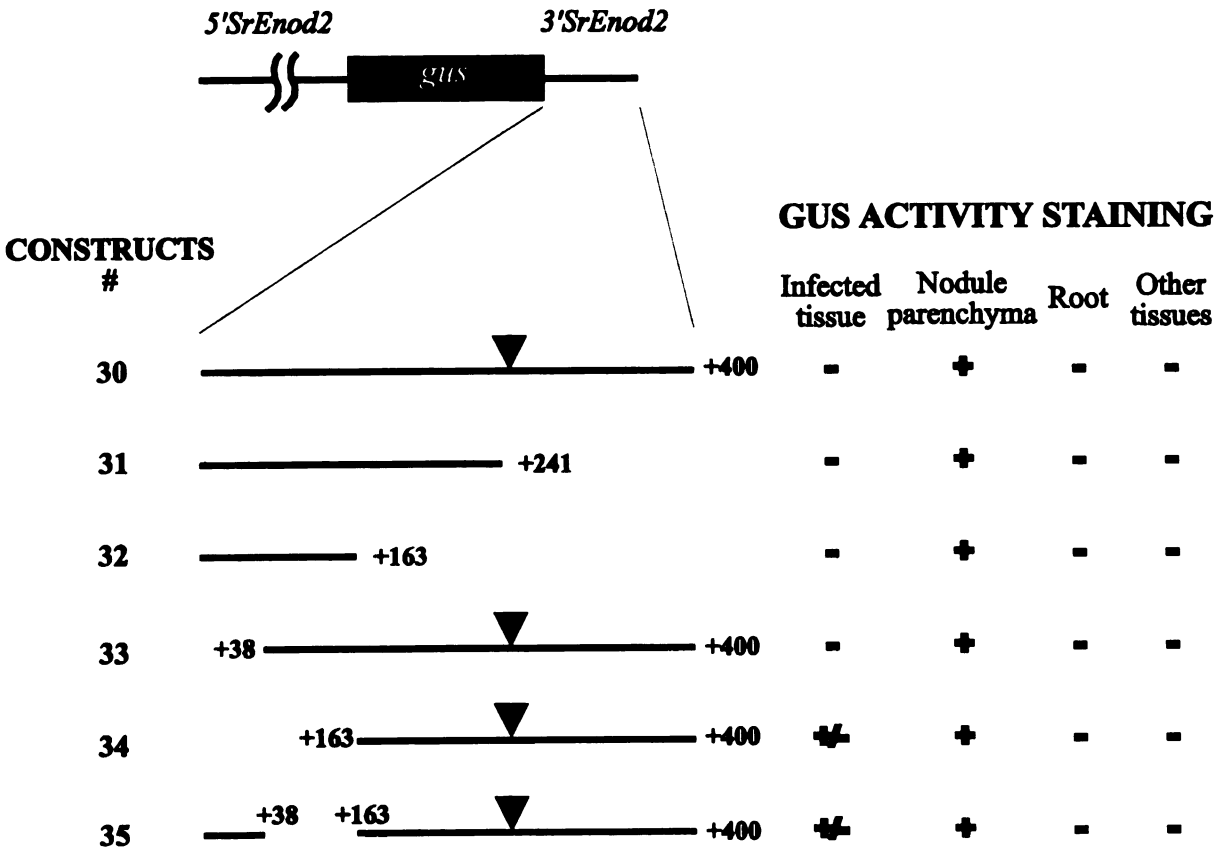
3' UTR were generated. The resulting fragments were fused to the *gus* reporter gene and the full-length *SrEnod2* 5' flanking region (Figure 3-3). The chimeric reporter gene fusions were introduced into *L. corniculatus* plants and their GUS expression was histochemically analyzed.

As summarized in Figure 3-3, a deletion from position +241 to position +163 maintains nodule parenchyma-specific expression (Construct 32), indicating that sequences between positions +1 and +163 are sufficient for tissue-specific expression. It should be noted that the potential poly(A) signal sequences between position +235 and +240 are deleted in this construct. However, other AATAAA-like sequences are present within this fragment. For examples, a canonical poly(A) signal sequence AATAAA is located at position +42, and two additional AATAAA-like sequences are present at positions +105 and +109, respectively (see Figure 3-2). It is likely that they may be recognized as alternative poly(A) signal sequences in order to form the 3'-end of the transcript.

A construct with a deletion in the *SrEnod2* 3' region from positions +1 to +38 still directed tissue-specific expression of the *gus* reporter gene (Figure 3-3, Construct 33). A construct with a deletion from +1 to +163 also directed nodule parenchyma-specific expression, but GUS activity could also be detected in the nodule-infected-tissues of some transgenic plants, indicating a less stringent tissue-specific control of gene expression (Figure 3-3, Construct 34). This data seems to be in contrast with the observation

LEGEND FOR FIGURE 3-3. Structure of *gus* fusion constructs and GUS expression in transgenic *L. corniculatus*.

The *gus* reporter gene was flanked by the *SrEnod2* 5' region and various 3' deletion and truncation fragments. The nucleotide numbers indicate the end-points of the deletion and truncation fragments, relative to the stop codon. The arrows represent the poly(A) sites. + and - indicate positive and negative GUS activity staining, respectively. Constructs 30 and 31 were previously analyzed (Chen et al., 1996; Chapter 2).


5'SrEnod2
3'SrEnod2
gus
CONSTRUCTS
#
GUS ACTIVITY STAINING
Infected tissue
Nodule parenchyma
Root
Other tissues
30
+400
31
+241
32
+163
33
+38
+400
34
+163
+400
35
+38
+163
+400

that sequences between positions +1 and +163 are sufficient for tissue-specific expression (see above). However, it is possible that redundant *cis*-elements may exist in the *SrEnod2* 3' UTR, and that deletion of some elements resulted in a slightly altered tissue-specific control. Support for this hypothesis came from the analysis of the expression pattern of a construct with a deletion of sequences between positions +38 and +163 (Figure 3-3, Construct 35), which was found to direct a similar reporter gene expression, namely in the nodule parenchyma, and in nodule central area of some transgenic plants. In constructs 33, 34 and 35, the potential poly(A) signals remain intact, suggesting that a normal polyadenylation process may take place. These results taken together suggest that sequences between positions +38 and +163 may be important for tissue-specific gene expression, and sequences between positions +163 and +400 may contain redundant *cis*-elements.

Proteins from nodule extracts interact with the RNA encoded by the 3' UTR of the *SrEnod2* gene

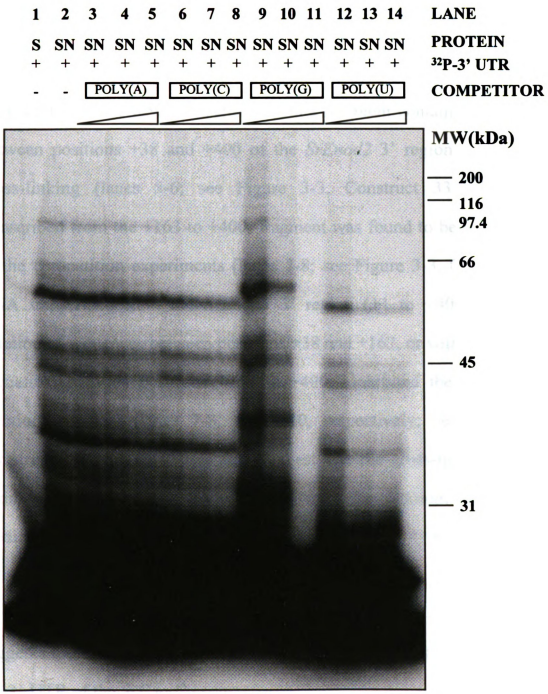
The *SrEnod2* gene is rapidly induced during early nodule development (Dehio and de Bruijn, 1992). To test whether proteins from nodule extracts could interact with the RNA encoded by the *SrEnod2* 3' UTR, total soluble proteins prepared from stem cortical tissues and 10-day old stem nodule

tissues, were UV-cross linked to ^{32}P-labeled sense and antisense RNAs generated by transcribing the 241 bp *SrEnod2* 3' UTR (see Methods). As shown in Figure 3-4, several protein bands from stem nodule extracts were found to be UV-cross linked to the sense RNA of the *SrEnod2* 3' UTR (lane 2). Some of the protein bands could also be detected in the UV-cross linking reaction with proteins from stem cortical tissues in a prolonged exposure, however, the intensity of labeling was extremely low (lane 1). In UV-cross linking experiments using the antisense RNA probe, proteins from both stem and stem-nodule tissues were cross-linked to the RNA, and the labeling was found to be profuse and identical in stem- and stem-nodule extracts, indicating a non-specific interactions between the antisense RNA and proteins from these tissues (data not shown).

Competition experiments using nonspecific competitors were carried out. As shown in Figure 3-4, UV-cross linking between stem-nodule proteins and the sense RNA probe was not affected by a 1000-fold excess of nonspecific competitors: poly(A) and poly(C) (lanes 3-5, and 6-8, respectively). The cross-linking was totally inhibited by a 1000-fold excess of poly(G) (Figure 3-4, lanes 9-11). Surprisingly, pre-incubation of the nodule protein extracts with small amounts of poly(G) shifted the UV-cross linked bands upwards, although a similar pattern was maintained (Figure 3-4, lanes 9-11). Pre-incubation of nodule protein extracts with poly(U)

LEGEND FOR FIGURE 3-4. RNA-protein UV-cross linking and competition with non-specific competitors.

Protein extracts (10 μg protein) of stem and 10-day-old stem nodule tissues were incubated with 125,000 cpm ^{32}P-labeled RNA derived from the 241 nt-long *SrEnod2* 3' UTR. Labeled proteins were visualized by autoradiography. Competition was performed by pre-incubation with 10-, 100-, and 1000-fold (w/w) non-specific competitors: poly(A), poly(C), poly(G), and poly(U), as indicated. S: stem; SN: stem nodule.

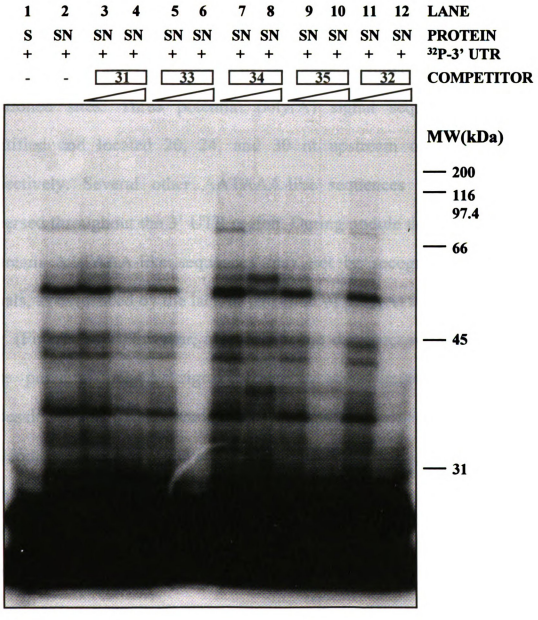

1 2 3 4 5 6 7 8 9 10 11 12 13 14
LANE
S SN SN SN SN SN SN SN SN SN SN SN SN SN
PROTEIN
+ + + + + + + + + + + + + +
32P-3' UTR
- - POLY(A) POLY(C) POLY(G) POLY(U)
COMPETITOR
MW(kDa)
200
116
97.4
66
45
31

inhibited the cross-linking reactions, but to a lesser extent (Figure 3-4, lanes 12-14).

Competition experiments using specific competitors were also carried out. As shown in Figure 3-5, the 241-nt RNA derived from the *SrEnod2* 3' UTR inhibited the cross-linking reactions (lanes 3-4; see Figure 3-3, Construct 31). The RNA transcribed from a fragment containing sequences between positions +38 and +400 of the *SrEnod2* 3' region inhibited the cross-linking (lanes 5-6; see Figure 3-3, Construct 33). The RNA transcribed from the +163 to +400 fragment was found to be less efficient in the competition experiments (lanes 7-8; see Figure 3-3, Construct 34). RNA transcribed from the *SrEnod2* 3' region (+1 to +400 bp) with a deletion of sequences between positions +38 and +163, or with a deletion of sequences between positions +163 to +400, inhibited the cross-linking reactions as well (lanes 7-8, and 9-10, respectively; see Figure 3-3, Constructs 35 and 32). These data suggest that the cross-linking between nodule proteins and the RNA derived from the *SrEnod2* 3' UTR are mediated by sequences located between positions +1 and +163, since deletion of these sequences results in a RNA that is inefficient in competition. Furthermore, the presence of multiple labeled bands also suggests that multiple proteins may interact with the RNA transcribed from the 3' UTR of the *SrEnod2* gene.

LEGEND FOR FIGURE 3-5. RNA-protein UV-cross linking and competition with specific competitors.

Protein extracts (10 μg protein) of stem and 10-day-old stem nodule tissues were incubated with 125,000 cpm ^{32}P-labeled RNA derived from the 241 nt-long *SrEnod2* 3' UTR. Labeled proteins were visualized by autoradiography. Competition was performed by pre-incubation with 10-, 100-fold (w/w) RNAs derived from the deletion and truncation fragments of the *SrEnod2* 3' UTR shown in Figure 3-3. S: stem; SN: stem nodule.


1 2 3 4 5 6 7 8 9 10 11 12 LANE
S SN SN SN SN SN SN SN SN SN SN SN PROTEIN
+ + + + + + + + + + + + 32P-3' UTR
- - 31 33 34 35 32 COMPETITOR
MW(kDa)
200
116
97.4
66
45
31

DISCUSSION

SrEnod2 mRNA 3'-end formation

S1 nuclease protection, 3' RACE and cDNA sequencing experiments reveal that *SrEnod2* transcripts appear to be polyadenylated at three closely-positioned sites. Three potential poly(A) signal sequences could be identified and located 20, 24, and 30 nt upstream of position +256, respectively. Several other AATAAA-like sequences were also found dispersed throughout the 3' UTR region. During nodule development, these upstream AATAAA-like sequences may not be recognized as poly(A) signals, as evidenced by the lack of detection of poly(A) addition near these sites (Figure 3-1). However, a reporter gene construct with a deletion of these potential poly(A) signal sequences still directed reporter gene expression to the nodule parenchyma (Figure 3-3, Construct 32), suggesting that some upstream poly(A) signal sequences may be alternatively used in the absence of these potential signal sequences.

Analysis of sequence motifs within the *SrEnod2* 3' UTR

Analysis of chimeric *SrEnod2-gus* reporter gene expression in transgenic plants indicates that sequences between positions +1 and +163 could direct

the *gus* reporter gene expression in the nodule parenchyma, suggesting that this sequence is sufficient to confer tissue-specific expression. This observation is supported by an altered reporter gene expression directed by constructs deleted of this sequence (Figure 3-3, Constructs 34 and 35). In these cases, reporter gene expression was found in nodule parenchyma and in the infected tissue of some transgenic plants. The reporter gene expression in the nodule central tissue is particularly interesting, because this suggests that the *SrEnod2* 5' region may have an extremely weak nodule-specific activity. The presence of the nodule parenchyma-specific *cis*-elements within the 3' UTR inhibits this weak activity in the infected tissue, and stimulates nodule parenchyma expression.

Another interesting observation is that nodule parenchyma expression remains observable in transgenic plants expressing constructs deleted of sequences between positions +1 and +163. This suggests that either redundant *cis*-elements exist in regions 3' to the position +163, or sequences between position +163 and +400 is a part of a large *cis*-element, which after truncation, is still able to direct nodule parenchyma expression.

Proteins that interact with the RNA encoded by the *SrEnod2* 3' UTR

Using *in vitro* protein-RNA UV-cross linking experiments, several proteins of different molecular weights interacting with the RNA transcribed from

the *SrEnod2* 3' UTR have been detected in nodule extracts. These interactions seem to be specific, since they can not be competed out by adding a 1000-fold excess of poly(A) and poly(C) homopolymers (Figure 3-4), but can be competed out by adding a 1000-fold excess of poly(G), and to a less extent poly(U), indicating that the sequences that interact with these proteins are GU-rich. Pre-incubation of nodule protein extracts with poly(G) polymers shifted cross-linking bands upwards. The reason for that is not clear. Furthermore, presence of multiple proteins interacting with the RNA derived from the *SrEnod2* 3' UTR suggests that they may interact with *cis*-elements within the *SrEnod2* 3' UTR to form a complex. Competition experiments with specific competitor RNAs derived from the *SrEnod2* 3' UTR suggests that sequences between positions +1 and +163 interact with nodule proteins. Thus, these *in vitro* data correlate nicely with the *in vivo* reporter gene expression patterns, suggesting that the sequences between positions +1 and +163, important for the nodule parenchyma expression, co-localize with the specific protein-binding activity. The significance of these interactions remains to be elucidated, but these findings open up possibilities for the isolation of tissue-specific *trans*-acting factors by screening cDNA expression libraries using riboprobes (For example, see Schumacher et al., 1995), or by using the yeast three-hybrid system developed by SenGupta et al. (1996).

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CHAPTER 4

THE *LjENOD2* GENE IS INDUCIBLE BY ETHYLENE IN ROOTS OF *LOTUS JAPONICUS*

ABSTRACT

The *SrEnod2* gene of *Sesbania rostrata* is induced during early nodule development, and also in *S. rostrata* roots treated with the plant hormone cytokinin in the absence of rhizobial infection. However, since *S. rostrata* is not amenable for molecular genetic analysis, we decided to study cytokinin regulation of nodulin gene expression and its involvement in *Lotus japonicus*, an emerging model legume. The *LjEnod2* gene was isolated and found to be inducible in roots of *L. japonicus*, but not by cytokinin. Submergence of the roots in water for several hours was sufficient to induce *Enod2* gene expression. Further analysis revealed that ethylene was the primary inducer. Application of ethylene inhibitors reduced the *Enod2* gene expression in roots. Pretreatment with the protein synthesis inhibitor cycloheximide abolished ethylene induction of *Enod2* gene expression, indicating that this process requires concurrent protein synthesis.

INTRODUCTION

The *Enod2* gene is an early nodulin gene, first isolated from soybean (Franssen et al., 1987). Its homologues have been isolated from other legumes, such as pea (van de Wiel et al., 1990a); alfalfa (van de Wiel et al., 1990b); lupine (Szczyglowski et al., 1992) and *Sesbania rostrata* (Dehio and de Bruijn, 1992). Expression of the *Enod2* gene in "empty-nodules" induced on alfalfa roots by *Rhizobium* nodulation-deficient mutant strains (*Nod*$^-$, van de Wiel et al., 1990b), as well as by strains deficient in exopolysaccharide synthesis (*Exo*$^-$; Dickstein et al., 1988), indicates that the *Enod2* gene product is not involved in the infection process *per se*, but rather in nodule morphogenesis.

The *Enod2* gene has also been found to be expressed in "pseudo-nodules" elicited by auxin transport inhibitors (Hirsch et al., 1989), or by a *Rhizobium* nodulation deficient (*Nod*$^-$) strain carrying a cytokinin biosynthetic gene *tzs* (Cooper and Long, 1994) on alfalfa roots. Plant hormones, therefore, seem to be involved in early nodule development (Cooper and Long, 1994; for a review, see Hirsch, 1992), as well as in early nodulin gene expression. Indeed, Dehio and de Bruijn (1992) found that the *Enod2* gene of *Sesbania rostrata* is induced specifically in roots after cytokinin treatment. Similar results were reported for alfalfa, although the

size of the cytokinin regulated transcript is larger than the corresponding transcript in nodules (Hirsch, et al., 1993).

Although the cytokinin up-regulation of *Enod2* gene expression in roots is not a common phenomenon in legume plants, the observation that the *SrEnod2* gene is specifically induced in roots of *S. rostrata* by cytokinins does provide a model for studying the mechanism of cytokinin regulated gene expression, as well as cytokinin involvement in nodule development. We have recently shown that the cytokinin up-regulation of the *SrEnod2* gene expression in roots of *S. rostrata* occurs at a post-transcriptional level, takes place mainly in the cytoplasm, requires protein synthesis, and appears to involve protein phosphorylation and dephosphorylation (Silver et al., 1996).

To study the developmental and tissue-specific regulation of *Enod2* gene expression, we have identified the 3' untranslated region of the *SrEnod2* gene as the minimal DNA sequence determinant responsible for nodule parenchyma-specific expression (Chen et al., 1996; Chapter 2). Therefore, we were interested in determining whether the same DNA sequence was responsible for the cytokinin up-regulation of *SrEnod2* gene expression. Unfortunately, we found that the *Enod2* genes of *L. corniculatus*, the plant used for our previous reporter gene expression studies, were not induced by cytokinin treatment. Therefore, the cytokinin

effects on *Enod2* gene expression could not be studied in *L. corniculatus* plants in parallel with the tissue-specific expression.

In a search for other transformable legumes where the *Enod2* gene was regulated in a developmental/tissue-specific manner, as well as by hormone treatment, we found *L. japonicus*. The *LjEnod2* gene of *L. japonicus* was isolated and found to be induced early during nodule development, and to be induced in roots in the absence of rhizobial infection, but not by cytokinin treatment. Rather, we found that incubating of roots in water was sufficient to induce *LjEnod2* gene expression. Here, we present experimental evidence in support of the notion that in *L. japonicus* roots, ethylene can induce the expression of the *Enod2* gene.

METHODS

Plant growth and treatment

Lotus japonicus Gifu B-129 seeds were surface sterilized and germinated according to Hansberg and Stougaard (1992). One-week-old seedlings were transferred into soil (vermiculite:sand, 6:1) and watered with B+D nutrient solution (Broughton and Dilworth, 1971) containing 0.5 mM KNO_3. Plants were grown in a growth cabinet (Conviron, Asheville, NC) at a 18 hr light 22°C and 6 hr dark 18 °C cycle, with a light intensity of 250 μE/sec/m^2 and

70 % RH. Two to three weeks old seedlings were gently washed free of soil and incubated in distilled water, containing the appropriate chemicals (Sigma, St. Louis, MO). 6-benzylaminopurine, aminoethoxyvinylglycine, silver thiosulfate, and cycloheximide were used at concentrations of 2, 100, 200, and 100 μM, respectively. To prepare silver thiosulfate, equal volumes of 0.2 mM silver nitrate and 1.6 mM sodium thiosulfate were mixed before use, according to Veen (1979). For ethylene treatments, seedlings were directly removed from soil, placed between water-soaked Whatmann papers (Schleicher & Schull, Keene, NH) and sealed in jars filled with either air or 20 ppm C_2H_4.

Isolation of cDNA clones and DNA sequence analysis

A nodule cDNA library of *L. japonicus* constructed using a oligo-dT primer in Lambda ZAPII vector (Stratagene Inc, La Jolla, CA), was kindly provided by Dr. Jens Stougaards, Aarhus, Denmark. Screening of cDNA clones was carried out according to standard procedures (Maniatis et al., 1989) and manufacturer's instructions. DNA sequences were determined by using the Sequenase 2.0 kit (United States Biochemical Inc., Cleveland, OH).

Southern blot analysis

Genomic DNA was isolated from leaves of *L. japonicus* using a CsCl ultracentrifugation protocol (Maniatis et al., 1989). 10 μg of DNA was digested with the BamHI, EcoRI, HindIII, SalI, XbaI, XhoI (Boehringer Mannheim, Mannheim, Germany), respectively, electrophorized in 0.8 % agarose gel. Gels were denatured and renatured according to standard methods (Maniatis et al., 1989) and blotted onto a Nylon membrane with a 0.45 μm pore size (Hybond-N; Amersham Life Science Inc., Arlington Heights, IL). Membranes were pre-hybridized and hybridized in 0.5 M phosphate buffer, pH 7.2, containing 7 % SDS and 1% BSA (Sigma) at 65^{o}C, according to Church and Gilbert (1984). An α ^{32}P-dATP-labeled DNA fragment containing the *SrEnod2* coding region was generated by random priming (Boehringer Mannheim) and used as a probe. Filters were washed at 65^{o}C in 2x SSC, 0.1 % SDS for 20 min; and 0.5x SSC, 0.1 % SDS for 20 min. Filters were exposed to X-ray films at -70^{o}C overnight.

Isolation of total RNA and northern blot analysis

Total RNA was isolated according the hot phenol method of Verwoerd et al., (1989), with minor modifications (Chen et al., 1996; Chapter 2). Ten micrograms of total RNA were electrophorized in 1 % (w/v) agarose gels in

1x MOPS buffer (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, pH 7) containing 7% (v/v) formaldehyde. Gels were soaked in 10x SSC solution for 30 min and blotted onto 0.22 μm nitrocellulose membranes (Nitro/Plus; MSI, Westborough, MA). Membranes were probed with an α ^{32}P-dATP-labeled *LjEnod2* cDNA fragment, generated by random priming (Boehringer Mannheim). Filters were re-probed with an HSP70 probe from *Arabidopsis* (Wu et al., 1988), an 18S rRNA DNA probe from rice (Takaiwa et al., 1984), or an ubiquitin probe from *S. rostrata* (kindly provided by M. Holsters, University of Gent, Belgium) as loading controls. All filters were washed at 65^{o}C in 2x SSC, 0.1% SDS for 20 min; 0.5x SSC, 0.1% SDS for 20 min; and 0.2x SSC, 0.1% SDS for 20 min. The signals were quantified using Phosphorimager analysis (Model 400B, Molecular Dynamics, Sunnyvale, CA).

RESULTS

cDNA isolation and DNA sequence analysis

The genomic organization of the *Enod2* genes in *L. japonicus* was analyzed by Southern blotting, using a ^{32}P-labeled DNA fragment containing the *SrEnod2* coding region (Dehio and de Bruijn, 1992). As shown in Figure 4-1, a single hybridizing band was found to be present in the BamHI, HindIII,

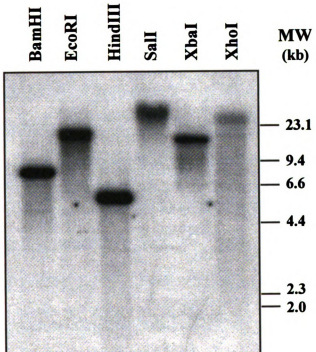
SalI and XbaI-digested genomic DNA, and two bands in the EcoRI-digested genomic DNA, suggesting that *L. japonicus* contains a single *Enod2* gene. To isolate a *LjEnod2* cDNA, a nodule cDNA library of *L. japonicus* was screened using the *SrEnod2* probe. A plasmid with a 1 kb insert was isolated and its DNA sequence was determined. The deduced amino acid sequence from this partial cDNA was found to contain proline-rich motifs (Figure 4-2), resembling those present in the *Enod2* proteins of other legumes (Franssen et al., 1987; van de Wiel et al., 1990a; Dehio and de Bruijn, 1992; Szczyglowski and Legocki, 1992). Northern blot analysis revealed that the size of the *Enod2* transcript in *L. japonicus* nodules was about 4 kb (see below).

Expression of the *LjEnod2* gene occurs at an early stage of nodule development

To examine the temporal expression of the *LjEnod2* gene during *L. japonicus* nodule development, root and nodule tissues were harvested at different time after rhizobial infection. Total RNA was isolated and subjected to northern blot analysis, using the ^{32}P-labeled partial *LjEnod2* cDNA as a probe. As shown in Figure 4-3, expression of the *LjEnod2* gene was induced between 7 and 10 days after infection, when nodule primordia were becoming visible on the roots. The expression was further enhanced to

LEGEND FOR FIGURE 4-1. Genomic organization of the *Enod2* gene in *Lotus japonicus* plants.

Genomic DNA of *L. japonicus* plants (10 μg/lane) was digested with BamHI, EcoRI, HindIII, SalI, XbaI, and XhoI, respectively, and separated by electrophoresis in a 0.8 % agarose gel. The blot was hybridized with a ^{32}P-labeled DNA fragment containing the *SrEnod2* coding region. A single hybridizing band was detected in digestions with all enzymes, except EcoRI, which gave two hybridizing bands. Molecular markers are indicated.


BamHI
EcoRI
HindIII
SalI
XbaI
XhoI
MW
(kb)
23.1
9.4
6.6
4.4
2.3
2.0

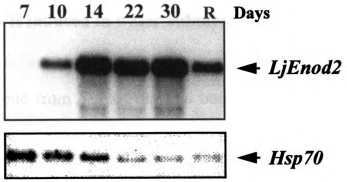
LEGEND FOR FIGURE 4-2. Nucleotide and the deduced amino acid sequences of a partial *LjEnod2* cDNA clone.

Number 1 is given to the first nucleotide at the 5' end of the insert. A star (*) indicates the stop codon. The poly(A) tail is in bold. A single letter designation is given to each amino acid deduced.

```
   1  GGCACGAGCCAACCTCCACCGTATGAGAACCCCCCACCAGTGCATCCGCCTCCTACACAT   60

      G  T  S  Q  P  P  P  Y  E  N  P  P  P  V  H  P  P  P  T  H
  61  CAGAAGCCATCACCAGGATACCAACCGCCTCATGTGAAACCACCACATATTGCGCCACCA  120

      Q  K  P  S  P  G  Y  Q  P  P  H  V  K  P  P  H  I  A  P  P
 121  CATGAAAAAACACCACCCAAATACCAACCACCTCATGAGAAGCCACCCTTTGAGAAGCCA  180

      H  E  K  T  P  P  K  Y  Q  P  P  H  E  K  P  P  F  E  K  P
 181  CCACTTGAGAAACCACCCCATGAAAAGCCACCACCAGAGTACCAACCTCCTCATAAGAAG  240

      P  L  E  K  P  P  H  E  K  P  P  P  E  Y  Q  P  P  H  K  K
 241  CCACCATCACAGTACCAACCACCACCAGAGTACGAACCTCCTCAGGAAAAACCACCACCT  300

      P  P  S  Q  Y  Q  P  P  P  E  Y  E  P  P  Q  E  K  P  P  P
 301  GTGTACCTACCCCCATATTGGAAACCACCACCTGCGTATCCACCTCCATATGAGAAACCA  360

      V  Y  L  P  P  Y  W  K  P  P  P  A  Y  P  P  P  Y  E  K  P
 361  CCACCAGAGTATGAACCGCCTCAAGAAAAACCACCACCAGTGTATTCGCCTCCATATGAA  420

      P  P  E  Y  E  P  P  Q  E  K  P  P  P  V  Y  S  P  P  Y  E
 421  AGGCCACCCACAGGGCATCCGACTCCTTACCCACCATTTTATCACCTACCACCTGTGTAT  480

      R  P  P  T  G  H  P  T  P  Y  P  P  F  Y  H  L  P  P  V  Y
 481  CACCCCCCTTACGAGAAGCCGCCACCACTATACCAACCTCCTCATGAGAAACCACCCATT  540

      H  P  P  Y  E  K  P  P  P  L  Y  Q  P  P  H  E  K  P  P  I
 541  TACGAACCTCCCCATGAGAAACCACCCATTTATGAACCTCCTCACGAGAAACCACCACTT  600

      Y  E  P  P  H  E  K  P  P  I  Y  E  P  P  H  E  K  P  P  L
 601  TATGAACCACCTCCAGGATACAACCCTCCACCATATGGTCACTATCCACCGTCTAAAAAT  660

      Y  E  P  P  H  E  K  P  P  I  Y  E  P  P  H  E  K  P  P  L
 661  TAATGACAACTACTAGTGAAGAACGTGGCAATGTGGCATGCATGTTTTGGTTAACCAAAA  720
      *  *

 721  GCAAGAGCAGTTCGTTTGTCACATAAATTTTCACTTTGTTTTTAATTTGATTCATGTTTA  780

 781  GGAAGCACTTTTAAGATGGGGAGTACAATACTCCACTTCTGCAATGGTTTGTAAAAATAA  840

 841  GGGCTCTCAATGCCTCTGTGTGTAATAATACCTTTCATTTCCGTTTCAAAAAAAAAAAA  900

 901  AAAAA
```

LEGEND FOR FIGURE 4-3. The *LjEnod2* gene is expressed early during nodule development.

Total RNAs were isolated from roots and nodules of *L. japonicus* at different times after rhizobial infection, as indicated by the numbers on top of the lanes. Total RNA was also isolated from roots submerged in water for 8 hr (lane labeled "R"). Ten micrograms of RNA were loaded in each lane and the blot was hybridized with a ^{32}P-labeled DNA probe prepared from the insert of the partial *LjEnod2* cDNA clone. The same blot was stripped and re-hybridized with a ^{32}P-labeled constitutively-expressed *Hsp70* DNA probe to calibrate sample loading.


7 10 14 22 30 R Days
LjEnod2
Hsp70

reach a plateau at day 14, when mature nodules had developed. The *LjEnod2* transcript level remained the same up to 30 days after infection. Thus, the temporal expression pattern of the *LjEnod2* gene in root-nodules of *L. japonicus* is similar to that of the *S. rostrata SrEnod2* gene during stem-nodule development, and different from the *SrEnod2* gene expression during root-nodule development, where it is expressed in a transient fashion (Dehio and de Bruijn, 1992).

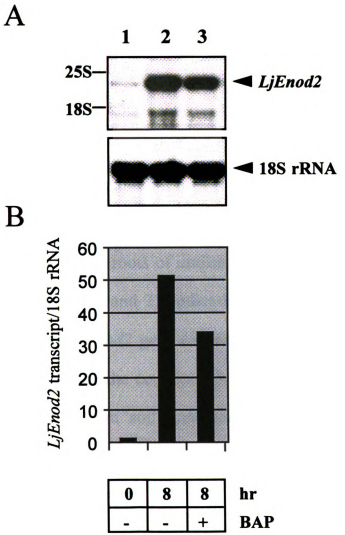
The *LjEnod2* gene is induced in water-submerged roots of *L. japonicus*

Since the *Enod2* gene from *S. rostrata* had been reported to be induced in roots treated with cytokinin (Dehio and de Bruijn, 1992; Silver et al., 1996), we examined whether the *LjEnod2* gene could also be induced in roots by cytokinin application. Two-week-old seedlings, with roots submerged in water containing 2 μM of benzylaminopurine (BAP) or lacking this hormone, were grown for 8 hr. Total root RNA was isolated and subjected to northern blot analysis. The result is shown in Figure 4-4. The *LjEnod2* transcript level was found to be greatly enhanced after 8 hr submergence in water (lanes 1 and 2). The *LjEnod2* transcript level in roots submerged in 2 μM BAP solution was also enhanced, but to a lower level (Figure 4-4, lane 3). These data indicate that submergence in water alone is sufficient to activate *LjEnod2* gene expression in roots of *L. japonicus*. This finding

LEGEND FOR FIGURE 4-4. *LjEnod2* gene expression in roots of *L. japonicus* plants treated with cytokinin.

(A) Northern blot analysis. Roots of two-weeks old seedlings were submerged in water solution containing 0 (lane 2) or 2 μM benzylaminopurine (BAP; lane 3) for 8 hr. Total RNAs were isolated and subjected to northern blot analysis with a ^{32}P-labeled *LjEnod2* probe. The primary LjEnod2 transcript is indicated with an arrowhead. The same blot was re-hybridized with a ^{32}P-labeled DNA probe encoding for the 18S rRNA to calibrate sample loading. The position of the ribosomal RNAs is also indicated.

(B) *LjEnod2* transcript levels relative to the 18S rRNA level. Relative *LjEnod2* transcript levels were calibrated with the 18S rRNA levels using Phosphorimager analysis. The RNA level in the root sample at time 0 was arbitrarily assigned the value 1.


A
1
2
3
25S
18S
LjEnod2
18S rRNA
B
LjEnod2 transcript/18S rRNA
60
50
40
30
20
10
0
0
8
8
hr
-
-
+
BAP

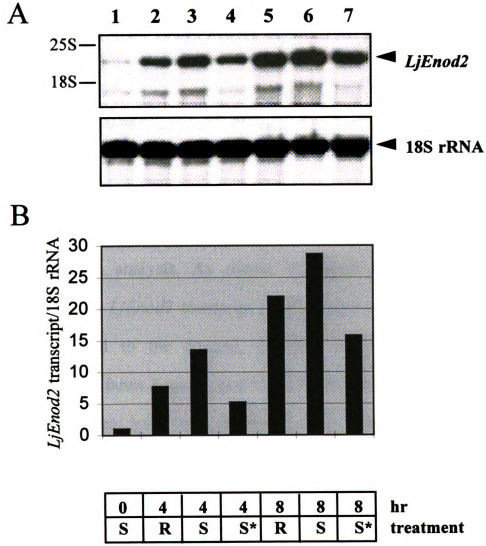
contrasts with the observation made with the *SrEnod2* gene, which has been shown to be specifically induced by cytokinin and not by submergence of roots in water (Dehio and de Bruijn, 1992). In fact, cytokinin does not only fail to induce the *LjEnod2* gene expression, but has a slightly negative effect on the level of the expression induced by water treatment (Figure 4-4, lanes 2 and 3).

To test whether the *Enod2* gene expression in roots was specific to submergence in water or to other side effects, such as wounding resulting from removing plants from soil, we tested the *Enod2* gene expression in seedlings grown undisturbed in pots. Additionally, to test whether the induction of the *Enod2* gene expression in roots required whole seedlings, we tested the *Enod2* gene expression in excised roots. As shown in Figure 4-5, the *LjEnod2* transcript in roots of undisturbed plants could be induced by water flooding (lanes 1, 4 and 7), indicating that water treatment alone sufficiently induced the *LjEnod2* gene expression. However, the induction was found to be smaller than the corresponding induction after submerging roots of plants removed from soil (compare lanes 3 and 4; 6 and 7), suggesting that other effects, such as wounding could enhance this induction. The *LjEnod2* transcript was also found to be induced in roots excised from the plants, indicating that the induction did not need whole seedlings (lanes 1, 2 and 5)

LEGEND FOR FIGURE 4-5. *LjEnod2* gene expression in roots of *L. japonicus* plants.

(A) Northern blot analysis. Three-weeks old seedlings were subjected to various treatments, such as excision of roots (R), removal from soil (S), before roots were submerged in water, or undisturbed in soil and pots being submerged (S*), for various time, as indicated at the bottom of panel B. Total RNA was isolated and subjected to northern blot analysis with a ^{32}P-labeled *LjEnod2* probe. The same blot was re-hybridized with a ^{32}P-labeled DNA probe encoding the 18S rRNA to calibrate sample loading. The position of the ribosomal RNAs is also indicated.

(B) *LjEnod2* transcript levels relative to the 18S rRNA level. Relative *LjEnod2* transcript levels were calibrated with the 18S rRNA levels using phosphorimager analysis. The RNA level in the root sample at time 0 was arbitrarily assigned the value 1.


A
1 2 3 4 5 6 7
25S
18S
LjEnod2
18S rRNA
B
LjEnod2 transcript/18S rRNA
30
25
20
15
10
5
0
0 4 4 4 8 8 8 hr
S R S S* R S S* treatment

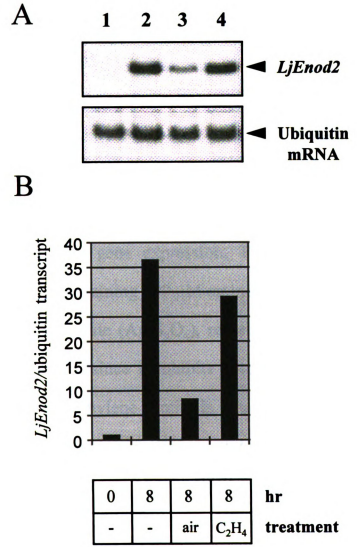
The *LjEnod2* gene is induced by ethylene in roots of *L. japonicus*

It has been observed that over-watering of *L. japonicus* plants causes accumulation of anthocyanines in the stems, indicating a stress response. It is also known that ethylene production in plants is induced by a variety of environmental factors, including wounding, anaerobiosis, chilling and water stress (Yang and Hoffman 1984; Abeles et al., 1992). To test whether ethylene was involved in the response of *LjEnod2* gene expression to water treatment, we determined *LjEnod2* transcript levels after the application of ethylene. Three-weeks old seedlings were removed from soil and placed between two layers of wet Whatmann paper and incubated in sealed jars containing air or ethylene (20 ppm) for 8 hr. Total RNA was isolated and subjected to northern analysis. As shown in Figure 4-6, the ethylene treatment induced the *LjEnod2* transcript level to approximately 3.5- and 29-fold, as compared to the control treatment and the initial level, respectively (compare lanes 3 and 4, and 1 and 4). In the control treatment, the *LjEnod2* transcript level was increased from the initial level to approximately 8-fold (Figure 4-6, lanes 1 and 3). In the same experiment, the *LjEnod2* transcript level was found to be induced from the initial level to approximately 36-fold by water submergence (Figure 4-6, lanes 1 and 2). Since wounding induces ethylene production (Yang and Hoffman 1984; Abeles et al., 1992), it is likely that the 8-fold induction in control samples

LEGEND FOR FIGURE 4-6. The *LjEnod2* gene expression in roots of *L. japonicus* plants upon ethylene treatment.

(A) Northern blot analysis. Three-weeks old seedlings were gently removed from soil and sealed in jars filled with either air or 20 ppm ethylene for 8 hr, as indicated at the bottom of panel B. Total RNAs were isolated and subjected to northern blot analysis with a ^{32}P-labeled *LjEnod2* probe. The same blot was re-hybridized with a ^{32}P-labeled DNA probe encoding an ubiquitin mRNA to calibrate sample loading.

(B) *LjEnod2* transcript levels relative to the ubiquitin mRNA level. Relative *LjEnod2* transcript levels were calibrated with the ubiquitin RNA levels using phosphorimager analysis. The RNA level in the root sample at time 0 was arbitrarily assigned the value 1.


A
1 2 3 4
LjEnod2
Ubiquitin mRNA
B
LjEnod2/ubiquitin transcript
40
35
30
25
20
15
10
5
0
0 8 8 8 hr
- - air C2H4 treatment

(air) is due to the action of ethylene produced in response to wounding. Taking this consideration into account, the ethylene treatment appears to induce the *LjEnod2* gene expression in roots of *L. japonicus* to a similar level as the water submergence treatment (Figure 4-6, lanes 2 and 4).

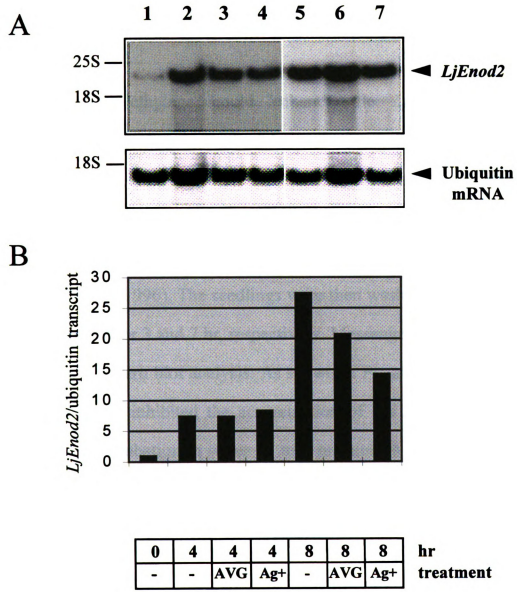
Inhibitors of ethylene biosynthesis and action reduce the induction of the *LjEnod2* transcript level

Since it appeared that ethylene could induce the *LjEnod2* gene expression in roots of *L. japonicus*, we examined whether application of inhibitors for ethylene biosynthesis and action could reduce the ethylene mediated induction of *LjEnod2* gene expression. Three-week old seedlings were incubated in water containing 100 μM aminoethoxyvinylglycine (AVG) and 0.2 mM silver thiosulfate ($Ag_2S_2O_3$), respectively. AVG is an inhibitor of ethylene biosynthesis, since it inhibits specifically ACC synthase, a rate-limiting enzyme for ethylene synthesis (Yu and Yang, 1979). Silver ion is an inhibitor of ethylene action (Beyer, 1976; 1979).Total RNA was isolated and subjected to northern blot analysis. As shown in Figure 4-7, the *LjEnod2* transcript level remained the same after 4 hr treatment with either AVG or $Ag_2S_2O_3$ (compare lanes 2, 3, and 4), although the ethylene production was inhibited by these treatments (data not shown). However, after 8 hr treatment with the inhibitors, the level of the *LjEnod2* transcript

LEGEND FOR FIGURE 4-7. *LjEnod2* gene expression in roots of *L. japonicus* plants treated with ethylene inhibitors.

(A) Northern blot analysis. Three-weeks old seedlings were treated with 100 μM AVG or 200 μM silver thiosulfate for various time, as indicated at the bottom of panel B. Total RNAs were isolated and subjected to northern blot analysis with a ^{32}P-labeled *LjEnod2* probe. The same blot was re-hybridized with a ^{32}P-labeled DNA probe encoding an ubiquitin mRNA to calibrate sample loading. The position of the ribosomal RNAs is also indicated.

(B) *LjEnod2* transcript levels relative to the ubiquitin mRNA level. Relative *LjEnod2* transcript levels were calibrated with the ubiquitin RNA levels using phosphorimager analysis. The RNA level in the root sample at time 0 was arbitrarily assigned the value 1.


A
1 2 3 4 5 6 7
25S
18S
LjEnod2
18S
Ubiquitin mRNA
B
LjEnod2/ubiquitin transcript
30
25
20
15
10
5
0
0 4 4 4 8 8 8 hr
- - AVG Ag+ - AVG Ag+ treatment

was reduced to 76 % and 52 % by the AVG and Ag^{+} treatment, respectively (compare lanes 5, 6, and 7). Silver ion treatment was found to be more effective than the AVG treatment, consistent with their mode of actions.

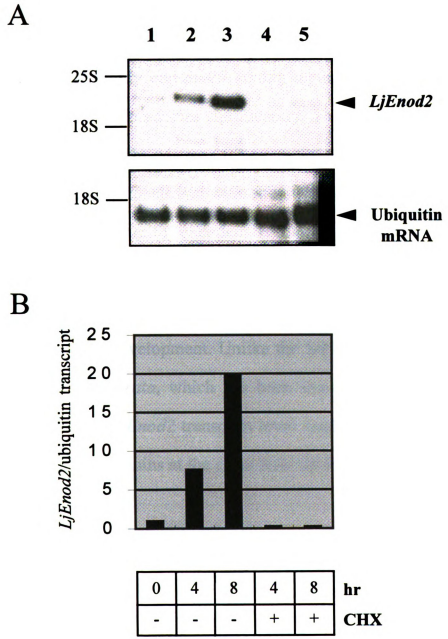
Cycloheximide inhibits the *LjEnod2* gene expression in roots

To test whether ethylene could enhance the *LjEnod2* transcript accumulation in the absence of translation, we monitored the *LjEnod2* transcript levels in roots after a pretreatment with a translational inhibitor cycloheximide. Three-week-old seedlings were pretreated for 1 hr with 100 μM cycloheximide, a concentration known to be effective for several plants (Silver et al., 1996). The seedlings were then washed extensively and incubated in water for 3 and 7 hr, respectively. Messenger RNA levels were determined by northern blot analysis. As shown in Figure 4-8, pretreatment with cycloheximide inhibited the accumulation of the *LjEnod2* transcript after subsequent incubation in water up to 7 hr (lanes 4 and 5), suggesting that *de novo* protein synthesis may be involved in the ethylene stimulation of the *LjEnod2* transcript accumulation.

LEGEND FOR FIGURE 4-8. *LjEnod2* gene expression in roots of *L. japonicus* plants treated with the protein synthesis inhibitor cycloheximide

(A) Northern blot analysis. Three-weeks old seedlings were pretreated with 100 μM CHX for 1 hr, and roots were submerged in water for additional 3 and 7 hr, as indicated as 4 and 8 hr CHX, respectively, at the bottom of panel B. Total RNAs were isolated and subjected to northern blot analysis with a ^{32}P-labeled *LjEnod2* probe. The same blot was re-hybridized with a ^{32}P-labeled DNA probe encoding an ubiquitin mRNA to calibrate sample loading. The position of the ribosomal RNAs is also indicated.

(B) *LjEnod2* transcript levels relative to the ubiquitin mRNA level. Relative *LjEnod2* transcript levels were calibrated with the ubiquitin RNA levels using phosphorimager analysis. The RNA level in the root sample at time 0 was arbitrarily assigned the value 1.


A
1 2 3 4 5
25S
18S
LjEnod2
18S
Ubiquitin mRNA
B
LjEnod2/ubiquitin transcript
25
20
15
10
5
0
0 4 8 4 8 hr
- - - + + CHX

DISCUSSION

The *LjEnod2* gene is highly conserved and expressed early during nodule development

Our analysis of the partial *LjEnod2* cDNA confirms that the coding region of *Enod2* genes is highly conserved among legume plants. However, the length of *Enod2* transcripts varies significantly. The *LjEnod2* gene encodes a transcript of about 4 kb; whereas for example, the *SrEnod2* transcript is about 1.4 kb. This is consistent with the notion that the nature of the repeating amino acid motifs, encoded by *Enod2* genes, are functionally more important than their length (van de Wiel et al., 1990a). Like the *Enod2* genes isolated from other legume plants, the *LjEnod2* gene is induced at an early stage of nodule development. Unlike the *SrEnod2* gene expression in root nodules of *S. rostrata*, which has been shown to be expressed in a transient fashion, the *LjEnod2* transcript level reaches a plateau at 14 days after nodulation, and remains at the same level up to 30 days.

The *LjEnod2* gene is induced by ethylene in roots

That ethylene appears to induce *LjEnod2* gene expression is surprising, but interesting. Ethylene has previously been implicated in nodulation, since

certain nodulation mutant phenotypes can be restored to wild-type by adding inhibitors of ethylene biosynthesis and action. For example, when the *sym5* pea mutant which nodulates poorly at 20^{o}C, is treated with AVG and Ag^{+}, the number of nodules formed is increased (Fearn and LaRue, 1991). It is also known that nitrate inhibits nodulation. This process may involve ethylene as well, since application of AVG releases nitrate inhibition of nodulation (Ligero et al., 1991). Moreover, the "thick and short roots" (Tsr) phenotype of *Vicia sativa* subsp. *nigra*, induced by its microsymbiont *Rhizobium leguminosarum* bv *Viciae*, can be suppressed by the ethylene inhibitor AVG (Zaat et al., 1989). Unlike other hormones, such as auxin and cytokinin, which have stimulating effects on nodulation, ethylene generally appears to have negative effects on early stages of nodule development (Lee and LaRue, 1992).

Even though the mechanism and significance of *LjEnod2* gene induction by ethylene remains to be elucidated. It is not known at present why the expression of *Enod2* genes from *S. rostrata*, *L. japonicus*, and other legumes such as soybean are regulated differently in roots, one by cytokinin and one by ethylene, while many others are not induced by these hormones. Clearly certain cytokinin effects on plant development can be coupled to ethylene responses. For examples, the observed cytokinin inhibition of root and hypocotyl elongation in dark-grown *Arabidopsis* seedlings mimics the plant response to ethylene (Cary et al., 1995). Application of the ethylene

inhibitor AVG partially releases the cytokinin inhibition (Cary et al., 1995). Furthermore, the coupling between cytokinin and ethylene mediated responses is evident in the cytokinin-resistant mutant *ckr1*, which is resistant to ethylene, and is allelic to the ethylene-resistant mutant *ein2* (Cary et al., 1995). Specific induction of *Enod2* genes by cytokinin versus ethylene may not involve a direct coupling between cytokinin and ethylene action. However, the two signal transduction pathways underlying their mode of action may overlap. The observation that the induction of *LjEnod2* gene expression by ethylene is inhibited by pretreatment with the protein synthesis inhibitor cycloheximide, suggests that *de novo* protein synthesis may be required for the ethylene mediated induction. In this respect, *LjEnod2* induction by ethylene is similar to the cytokinin up-regulation of the *SrEnod2* gene expression (Silver et al., 1996).

The *LjEnod2* gene is the first nodulin gene that has been shown to be ethylene-inducible. This may provide a tool to study ethylene involvement in nodule development and a tool to study regulation of other nodulin genes by ethylene.

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CHAPTER 5

FUNCTION OF THE ENOD2 PROTEIN IN NODULES

ABSTRACT

The *Enod2* gene encodes a putative cell wall protein, and *Enod2* transcripts are localized in the nodule parenchyma, which has been hypothesized to function as a barrier for oxygen diffusion. To examine the proposed role of nodulin Enod2 in the formation of this oxygen barrier, we attempted to down-regulate *Enod2* gene expression in transgenic *Lotus corniculatus* plants by expressing antisense *Enod2* constructs. In transgenic plants, the endogenous *Enod2* transcript levels were found to be altered, but not completely abolished. Expressing the antisense *Enod2* constructs did not affect plant growth and nodulation, and nodules were found to be able to reduce acetylene, indicating the presence of a functional nitrogenase enzyme. However, nodules formed on several transgenic antisense plants appeared to be smaller than those on control plants, indicating an alteration in nodule morphogenesis. Oxygen microelectrode measurements revealed that the oxygen diffusion profile of some transgenic plants may be altered.

INTRODUCTION

Oxygen plays an important role in symbiotic nitrogen fixation. On the one hand, the nitrogenase activity requires high energy (ATP) input, which has to be generated by bacterial oxidative phosphorylation in the nodule infected cells. On the other hand, the nitrogenase complex is irreversibly and rapidly inactivated by oxygen (Robson and Postgate, 1980). This apparent oxygen paradox is reconciled by the creation of a microaerobic environment inside of nodules. Direct measurements of nodule internal oxygen concentration (O_i) have provided evidence for the presence of physical, as well as variable oxygen diffusion barriers in nodules, to allow a continuous flow of oxygen to the infected cells of the nodule, while maintaining the O_i at a low level to prevent nitrogenase from inactivation.

Using oxygen microelectrodes, Tjepkema and Yocum (1974) were able to measure the free oxygen concentration in nodules of soybean (*Glycine max* Merr.). They found that the O_2 concentration decreased sharply across the nodule cortex and was below the detection level in central tissues. These data suggested that the resistance to O_2 diffusion into the nodule primarily occurred within the cortex. Subsequently, Witty et al. (1987) confirmed this observation in nodules of pea (*Pisum sativum*) and French bean (*Phaseolus vulgaris*). Detailed microscopic examination of the nodule anatomy revealed that the inner cortex (named nodule parenchyma

thereafter) has compact and small cells, with fewer intercellular spaces than in other nodule tissues (van de Wiel et al., 1990). Since oxygen diffusion is approximately four orders of magnitude slower in solution than in air, the nodule parenchyma, therefore has been hypothesized to be the site of resistance to oxygen diffusion in nodules (van de Wiel et al., 1990).

A nodule variable resistant barrier to oxygen has been identified in experiments, in which the nitrogenase was found to be active in nodules exposed to higher rhizosphere O_2 concentration (Sheehy et al., 1983; for a review, see Hunt and Layzell, 1993) The change in resistance to O_2 has been linked to inhibition of nitrogenase activity by a number of environmental and physiological factors (for a review, see Hunt and Layzell, 1993). However, the site of the variable barrier and its relationship to the physical barrier are currently not well understood.

The expression of the *Enod2* gene has been localized to the nodule parenchyma (Nap and Bisseling, 1990; van de Wiel et al., 1990). Since it is a putative cell wall protein, the Enod2 protein has, therefore, been postulated to contribute to the special morphology of the nodule parenchyma, and the formation of the oxygen diffusion barrier (van de Wiel et al., 1990). To test this hypothesis, we have used a genetic approach to down-regulate *Enod2* gene expression in transgenic *L. corniculatus* plants, by expressing antisense *Enod2* transcripts. We have generated antisense expressing constructs using both a strong constitutive promoter and a

nodule parenchyma-specific promoter. Here, we report the phenotypic and physiological analysis of these transgenic plants.

METHODS

Plant transformation and growth

L. corniculatus plants were grown in soil mix (Vermiculite:sand:MetroMix, 5:3:1) in pots maintained in a controlled environment cabinet (Conviron, Asheville, NC) at 24°C during the day and 20°C at night, with a 16 hr photoperiod (250 μE/sec/m2). For nitrogenase activity measurements, six cuttings of each plant to be analyzed were generated and grown for one week to regenerate roots. These plants were inoculated with a two-day-old culture of *Rhizobium loti* NZP 2037 (Pankhurst, 1983) and grown for two weeks. Plants were subsequently repotted into silica sand, in sealable pots, and grown for two weeks. During this growth period, plants were watered with B+D nutrient solution (Broughton and Dilworth, 1971), containing 0.5 mM KNO_3.

Isolation of total RNA and northern blot analysis

Total RNA was isolated following the hot phenol method of Verwoerd et al., (1989), with modifications described previously (Chen et al., 1996; Chapter 2). Northern blot analysis was carried out following standard procedures (Maniatis et al., 1989). A α^{32}P-dATP-labeled DNA fragment containing the *SrEnod2* coding region was used as a probe.

Antisense *Enod2* constructs preparation

A 1.3 kb MspI fragment, containing the 5' UTR and most of the *SrEnod2* coding region (Dehio and de Bruijn, 1992), was cloned into the AccI site of pUC18 (GIBCO BRL, Gaitherburg, MD) to generate plasmid pCD21 (Dehio and de Bruijn, 1992). To generate the antisense *Enod2* construct containing coding sequences (Figure 5-1, Construct 37), a 900-bp BamHI-SpeI fragment was excised and inserted into the BamHI-SpeI sites of the binary vector pROK2275 (Szabados et al., 1990), harboring a modified polylinker between the CaMV35S promoter and the *gus* coding region (AccI-BamHI-SalI-SmaI-SpeI). To generate the antisense *Enod2* construct containing primarily 5' sequences (Figure 5-1, Construct 36), a 400-bp SpeI-PstI fragment was excised from pCD21 and inserted into the XbaI-PstI sites of pUC18. The resulting plasmid was digested with PstI and blunt-

ended with T4 DNA polymerase (Maniatis et al., 1989). The linearized plasmid was digested with BamHI, and the 400 bp resulting fragment was purified and inserted into the BamHI and the blunt-ended SalI sites of the modified binary vector pROK2275. To generate the antisense *Enod2* construct containing primarily 3' sequences (Figure 5-1, Construct 38), a 280-bp BamHI-KpnI fragment was excised from pCD22A (Dehio and de Bruijn, 1992), and cloned into the BamHI-KpnI sites of pROK2275. To generate the antisense *Enod2* constructs without the *gus* gene, the 1.3 kb BamHI and blunt-ended HindIII fragment of pCD21 was cloned into the BamHI and blunt-ended SacI sites of the binary vector pBI121 (Clontech Laboratories, Palo Alto, CA) (Figure 5-1, Construct 39), or the binary vector *5'SrEnod2-gus-3'SrEnod2* (Figure 5-1, Construct 40; see Chapter 2).

Oxygen microelectrode measurement of nodule oxygen tensions

Oxygen microelectrode measurements were carried out essentially as described by Tjepkema and Yocum (1974) with the following modifications. Briefly, nodules were detached from roots 21-28 days after nodulation and embedded in 0.5 % agarose positioned on top of a layer of solidified 1 % agarose in a 1 cm^3 chamber. Nodules were positioned in such a way that the apical region points to the side of the chamber and that the nodules were 1 to 1.5 cm under the surface. An oxygen electrode (ca. 10 μm

in tip width; kindly provided by Dr. David Emerson, Michigan State University, MI) was used to monitor the oxygen tension.

Measurement of nitrogenase activity using an open-flow gas exchange system

The open-flow gas exchange system developed by Hunt et al., (1989) was utilized to measure nitrogenase activity. Briefly, nodulated plants were grown in silica sand for two weeks and the pots were sealed one day before gas exchange. A computer-controlled mixture of gases was passed through the pots and both H_2 and CO_2 evolution in the efflux was measured using a H_2 analyzer (Model H-150, Morgen Instruments, Andover, MA) and an infrared CO_2 analyzer (Model 225, Mark III, Analytical Development Corporation, Hoddesdon, U.K.). Apparent nitrogenase activity (ANA) was defined as the level of H_2 evolution in air. Total nitrogenase activity (TNA) was defined as the level of H_2 evolution in $Ar:O_2$. Potential nitrogenase activity (PNA) was defined as the maximum rate of H_2 evolution in $Ar:O_2$, during a linear increase in pO_2 from 20 to 100 % in a 30-min period (Diaz del Castillo et al., 1992). For dry weight measurement, nodules were first dehydrated in a 80^oC baking oven for two days and then weighed.

RESULTS

Generation of antisense *Enod2* constructs

Three antisense *Enod2* constructs were generated using fragments covering the 5', internal and 3' portions of the *SrEnod2* genomic locus (Figure 5-1, Constructs 36, 37, and 38; Dehio and de Bruijn, 1992). To facilitate the detection of expression of these antisense constructs, these fragments were fused, in an antisense orientation, to a bacterial *gus* reporter gene (Jefferson, 1987). A strong promoter from CaMV35S (Benfey et al., 1989) was employed to generate antisense transcripts. The constructs were introduced into *L. corniculatus* plants by *A. rhizogenes*-mediated plant transformation (Szabados, et al., 1990). The expression of the antisense transcripts was monitored in roots of transgenic plants by GUS activity staining, and found to be positive in 12 out 30, 7 out of 23, and none out of 16 plants, harboring the antisense constructs containing the 5', 3' and the coding sequences, respectively (data not shown). The other two antisense constructs (Figure 5-1, Constructs 39 and 40) were constructed such that a DNA fragment containing the 5' and internal portions of the *SrEnod2* gene was fused in an antisense orientation to either the CaMV35S promoter or the *SrEnod2* 5' and 3' sequences responsible for directing expression to the nodule

LEGEND FOR FIGURE 5-1. Antisense *Enod2* constructs.

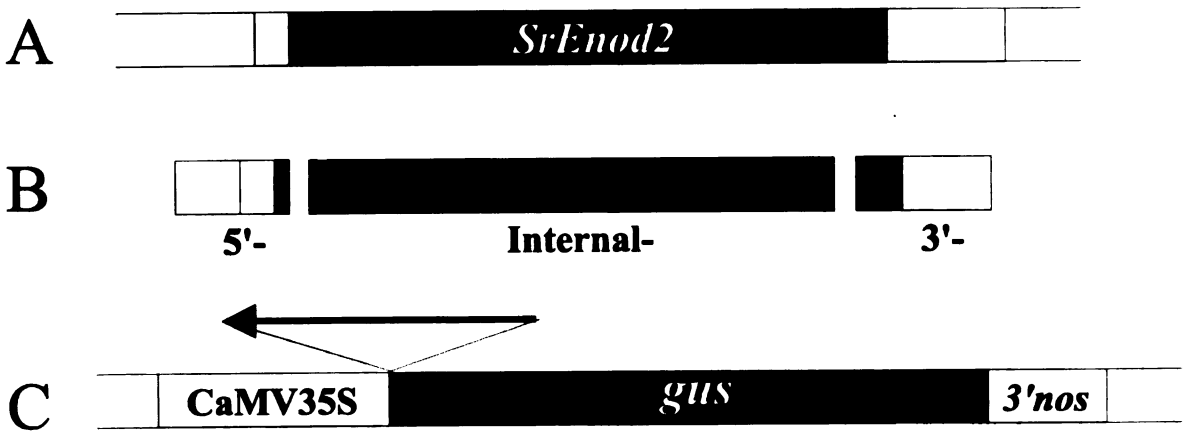
(A) The *SrEnod2* gene locus. The black box represents the *SrEnod2* coding region. Open boxes at each end of the black box represents the 5' and 3' untranslated regions of the *SrEnod2* gene.

(B) Subfragments of the *SrEnod2* gene. 5'-, internal-, and 3'- indicate subfragments corresponding to the 5', internal, and 3' portions of the *SrEnod2* gene, respectively.

(C) The binary vector used to generate the 5'-, Internal-, and 3'- antisense *Enod2* constructs. The insertion site and orientation are indicated by an arrow. The construct numbers and description are provided.

(D) A antisense *Enod2* construct. The fragment containing the 5' and internal portions of the *SrEnod2* gene was inserted between the CaMV35S promoter and the 3' *nos* terminator. The construct number and description are provided.

(E) A antisense *Enod2* construct. The fragment containing the 5' and internal portions of the *SrEnod2* gene was inserted between the *SrEnod2* 5' and 3' flanking sequences (Chen et al., 1996; see Chapter 2). The construct number and description are provided.



Construct 36: 5'-Antisense
Construct 37: Internal-Antisense
Construct 38: 3'-Antisense

Construct 39: 35S-Antisense

Construct 40: SrEnod2-Antisense

parenchyma (Chen et al., 1996; see Chapter 2). 40 and 80 transgenic plants harboring these two antisense constructs, respectively, were generated.

Phenotype of antisense *Enod2*-expressing transgenic plants

The majority of the 69 transgenic *L. corniculatus* plants harboring the antisense constructs 36, 37, and 38, as well as 8 plants transformed with the control construct, 5'*Srglb3-gus-3'nos* (designated as pLP32; Szabados, et al., 1990), appeared to be normal as compared to wild type plants. However, a couple of plants harboring the 5'-antisense *Enod2* construct (Figure 5-1, Construct 36), showed severely altered phenotypes (reduced internode length and early senescence of leaves). The reason for that is not clear. One possibility is that the expression of *rolABC* genes (White et al., 1985) from the *Agrobacterium rhizogenes* strain used to transform *L. corniculatus* plants in these experiments caused the observed plant growth phenotypes, since tobacco plants expressing these genes showed similar phenotypes (Sinkar et al., 1988; Estruch et al., 1991a; b). However, the possibility that the expression of the 5'-antisense *Enod2* transcript caused the phenotype can not be completely ruled out.

Transgenic plants were also tested for their nodulation characteristics. All plants, including those plants showing severe phenotypes, could be effectively nodulated. Nodules were pink, indicating that they were able to

fix nitrogen. This was confirmed by monitoring nitrogenase activity using the acetylene reduction assay (data not shown). When transgenic plants with altered endogenous *Enod2* transcripts levels (see below) were chosen to measure their nodule fresh weight and number, nodules formed on several of these plants were found to be smaller than those formed on the wild type and control plants (Figure 5-3).

Endogenous *Enod2* mRNA levels in transgenic antisense plants

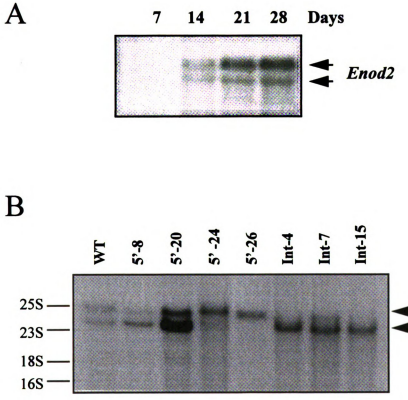
Endogenous *Enod2* mRNA levels in nodules of transgenic antisense plants were examined by Northern blot analysis. Two major *Enod2* transcripts were found in wild type nodules (Figure 5-2, Panel A). Transgenic antisense plants were found to contain various levels of these two transcripts (Figure 5-3, Panel B). A total disappearance of the two endogenous *Enod2* transcripts was not observed in 69 individual transgenic plants harboring the three different antisense constructs (Figure 5-1, Constructs 36, 37, and 38; data not shown). Similar results were obtained after analyzing 120 transgenic plants transformed with the other two constructs (Figure 5-1, Constructs 39 and 40; data not shown).

Accumulation of the antisense transcripts was also examined in the transgenic plants, and the antisense transcripts could not be detected. Since the activity of the GUS fusion protein had been detected in transgenic

LEGEND FOR FIGURE 5-2. The *Enod2* gene expression in *L. corniculatus* plants.

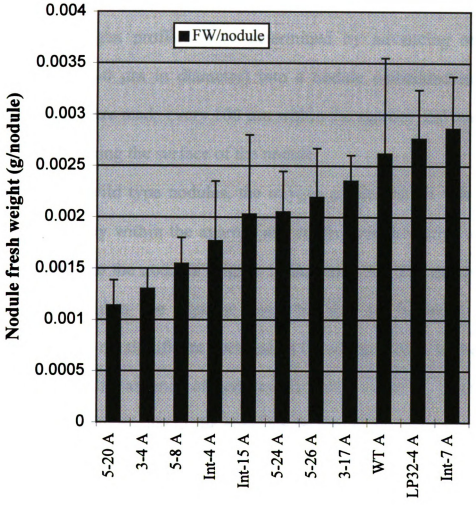
(A) Northern blot analysis. The *Enod2* gene expression during nodule development of wild type *L. corniculatus* plants is shown. Two major transcripts were detected 14 days after rhizobial infection.

(B) Northern blot analysis. The endogenous *Enod2* gene expression in transgenic plants harboring the 5'- and Internal- antisense *Enod2* constructs is shown. The position of ribosomal RNAs is indicated.


A
7
14
21
28
Days
Enod2
B
WT
5'-8
5'-20
5'-24
5'-26
Int-4
Int-7
Int-15
25S
23S
18S
16S
Enod2

LEGEND FOR FIGURE 5-3. Nodule fresh weight of transgenic *L. corniculatus* plants harboring antisense *Enod2* constructs.

Nodules formed between 4 and 5 weeks after rhizobial infection were collected and weighed. Six replicates for each transgenic line were assayed. The solid black boxes represent average values, and the error bars represent the standard deviation.


FW/nodule
Nodule fresh weight (g/nodule)
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
5-20 A
3-4 A
5-8 A
Int-4 A
Int-15 A
5-24 A
5-26 A
3-17 A
WT A
LP32-4 A
Int-7 A

plants, it is possible that the antisense transcripts were not stable, or the level of expression was low in these transgenic plants.

Oxygen microelectrode measurements of nodule O_2 tension

The nodule oxygen profiles were determined by advancing an oxygen microelectrode (10 μm in diameter) into a nodule embedded in agarose. Measurements were made every 100 μm within the agarose and every 50 μm after reaching the surface of the nodule.

In case of wild type nodules, the oxygen concentration was found to decrease gradually within the agarose and to drop sharply after advancing 50 to 100 μm into the nodules (Figure 5-4A, Panel A). The decrease in O_2 concentration within the agarose may be due to bacterial or plant respiration, since no significant decrease in O_2 concentration in the agarose was observed in the absence of nodules. The drop in the O_2 concentration inside nodules may be due to the presence of the O_2 barrier, as reported by Tjepkema and Yocum (1974). We observed that the O_2 concentration on the surface of nodules remained relatively constant for nodules derived from the same plant, this phenomenon may reflect a balance between nodule respiration and oxygen diffusion capacity. The O_2 concentration on the surface of wild type nodules was found to be 135.7 +/- 11.4 μM (Figure 5-4B), while the O_2 concentration on the surface of nodules derived from

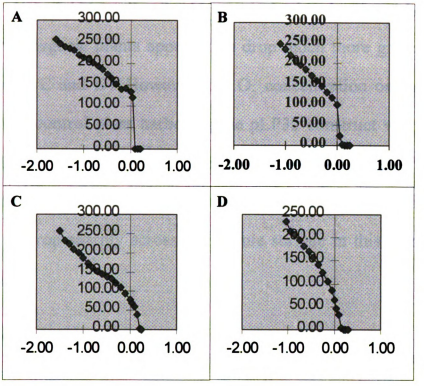
LEGEND FOR FIGURE 5-4. Oxygen microelectrode measurement of O_2 concentrations across nodules of transgenic plants expressing antisense *Enod2* transcripts.

Nodules three- to four-weeks of age were excised from both wild type plants and transgenic plants harboring the 5'- antisense construct (Figure 5-1, Construct 36), and embedded in 0.5 % agarose. Oxygen concentration within the agarose and across the nodule were measured in a step-wise fashion. Measurements were made from 1 to 6 nodules. For each nodule, three to four measurements were made.

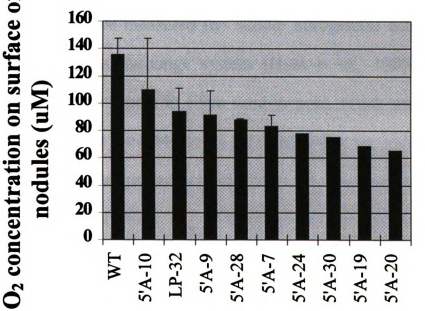
(A) The pO_2 profiles of nodules. The oxygen profiles of a representative nodule of the wild type (Panel A), a control plant harboring the pLP32 construct (Panel B), and two transgenic plants (Panel C: 5'-24, Panel D: 5'-20) were shown. The Y axis represents oxygen concentration in μM, and the X axis represents the distance (cm) from the nodule surface, as indicated by the cross-point of the Y and X axis.

(B) The O_2 concentration on the nodule surface. The O_2 concentration on the surface of nodules of transgenic plants expressing the 5'- Antisense transcript was shown. The solid black boxes represent average values, and the error bars represent the standard deviation.

A


A
300.00
250.00
200.00
150.00
100.00
50.00
0.00
-2.00 -1.00 0.00 1.00
B
300.00
250.00
200.00
150.00
100.00
50.00
0.00
-2.00 -1.00 0.00 1.00
C
300.00
250.00
200.00
150.00
100.00
50.00
0.00
-2.00 -1.00 0.00 1.00
D
250.00
200.00
150.00
100.00
50.00
0.00
-2.00 -1.00 0.00 1.00

B


O2 concentration on surface of nodules (uM)
160
140
120
100
80
60
40
20
0
WT
5'A-10
LP-32
5'A-9
5'A-28
5'A-7
5'A-24
5'A-30
5'A-19
5'A-20

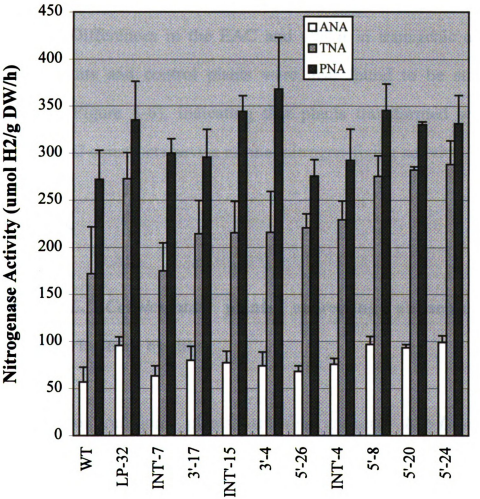
several transgenic plants was found to be much lower than that of wild type (Figure 5-4B). Additionally, the O_2 concentration inside nodules of these particular transgenic plants appeared to drop much more gradually (Figure 5-4A, Panels C and D). However, the O_2 concentration on the surface of nodules of a control plant harboring the pLP32 construct was found to be lower than that of wild type nodules (93.79 +/- 17.17 μM; Figure 5-4B). The reason for this observation is not clear. Still, the O_2 concentrations appeared to drop sharply across the nodule surface in this plant (Figure 5-4A, Panel B).

Nitrogenase activity in nodules of transgenic antisense plants

To assess the effect of expressing antisense *Enod2* transcripts on nitrogenase activity, we measured the nodule nitrogenase activity using a non-invasive open gas exchange system (Hunt et al., 1989). Nodulated transgenic plants were grown in silica sand in pots, which were sealed for gas exchange analysis (see Methods). Six replicates for each plant were analyzed. Apparent nitrogenase activity (ANA, H_2 production in air); total nitrogenase activity (TNA, H_2 production in $Ar:O_2$); and potential nitrogenase activity (PNA, maximum H_2 production in $Ar:O_2$ as pO_2 is increased from 20 % to 100 %) were measured simultaneously. Based on the resulting data, the electron allocation coefficient (EAC=1-ANA/TNA;

LEGEND FOR FIGURE 5-5. Measurement of nitrogenase activity.

Apparent nitrogenase activity (ANA), total nitrogenase activity (TNA), and potential nitrogenase activity (PNA) were measured in an open-flow gas exchange system (Hunt et al., 1989). Plants 4 to 5 weeks after rhizobial infection were analyzed and six replicates for each transgenic line were tested. The black, gray, and white columns represent the average values. The Error bars represent the standard deviation.


Nitrogenase Activity (umol H2/g DW/h)
450
400
350
300
250
200
150
100
50
0
ANA
TNA
PNA
WT
LP-32
INT'-7
3'-17
INT'-15
3'-4
5'-26
INT'-4
5'-8
5'-20
5'-24

proportion of electrons allocated to N_2 fixation) and the oxygen limitation coefficient of nitrogenase (OLCn=TNA/PNA; index of degree to which O_2 supply limits nitrogenase activity) were calculated. On a nodule dry weight basis, the nitrogenase activities appeared to be qualitatively the same in nodules of transgenic antisense-expressing plants versus control plants (Figure 5-5). Differences in the EAC and OLCn in transgenic antisense-expressing plants and control plants were also found to be statistically insignificant (Figure 5-6), indicating that plants transformed with these antisense *Enod2* constructs have a normal nitrogen-fixing activity.

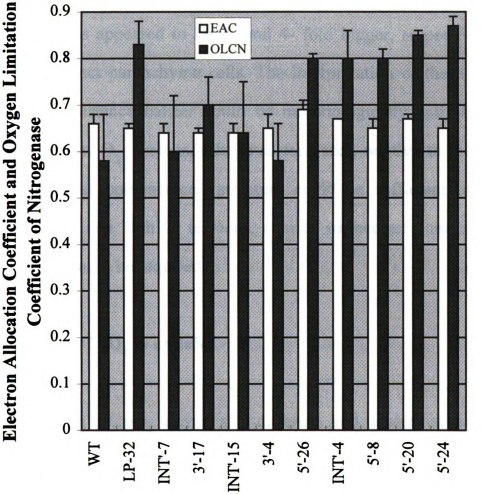
DISCUSSION

Transgenic *L. Corniculatus* plants expressing antisense *Enod2* transcripts form small nodules

The majority of transgenic plants harboring antisense *Enod2* constructs grew normally and could be efficiently nodulated. However, several antisense transgenic plants formed nodules smaller than the wild type (Figure 5-3), indicating an alteration in nodule morphogenesis in these transgenic plants. The molecular mechanism underlying the small nodule phenotype is not known. However, it has been reported that nodule morphogenesis can be modified by exposure to different levels of ambient

LEGEND FOR FIGURE 5-6. Electron allocation and oxygen limitation coefficients of nitrogenase.

The electron allocation coefficient of nitrogenase was calculated based on the equation (EAC=1-ANA/TNA). The oxygen limitation coefficient of nitrogenase was calculated based on the equation (OLCn=TNA/PNA). The black and white columns represent the average values of six independent measurements. The error bars represent the standard deviation.


Electron Allocation Coefficient and Oxygen Limitation Coefficient of Nitrogenase
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
EAC
OLCN
WT
LP-32
INT'-7
3'-17
INT'-15
3'-4
5'-26
INT'-4
5'-8
5'-20
5'-24

oxygen concentration. For example, nodules of cowpea were able to adapt through morphological and structural changes to function under sub- and supra-ambient oxygen pressures (Dakora and Atkins, 1990). When cowpea plants were grown in 1% oxygen, nodules appeared to be smaller with small but round parenchyma cells. When plants were grown in 20 % and 80 % oxygen, nodules appeared to be 2- and 4- fold bigger, respectively, with long and compact parenchyma cells. The interpretation of these data was that the O_2 resistance barrier could be morphologically and structurally modified. At low O_2 tensions, a large surface area, relative to the infected cells, and a less compact cell structure could be sufficient to provide resistance to O_2. At high O_2 tensions, a small surface area and a compact cell structure would be required.

Measurement of pO_2 in nodules

The monitoring of pO_2 across the different cell layers of nodules of transgenic plants expressing antisense *Enod2* transcripts, suggests that the pO_2 across nodules of several transgenic plants dropped gradually, as opposed to sharply, as observed in control nodules (Figure 5-4A). Although, a detailed microscopic study would be necessary to correlate the cell structures of these transgenic nodules with the altered profile of pO_2, the

alteration in the pO_2 profile, nevertheless, suggests that resistance to O_2 in nodules of these transgenic plants may be modified.

Nitrogenase activity in nodules of transgenic plants expressing antisense *Enod2* transcripts

The nitrogenase activity (ANA; TNA; and PNA) measurements revealed that there were no significant differences between transgenic antisense plants and control plants. These studies are consistent with previous observations that legume plants can modify their nodule structures to adapt different rhizosphere pO_2 environments (Dakora and Atkins, 1990). Additionally, the presence of a physical diffusion barrier may not be the only mechanism that was evolved in nodules of legume plants to prevent nitrogenase from inactivation. For examples, several proline-rich proteins have been identified in the extracellular space of nodule cortex (Sherrier and VandenBosch, 1994), and a glycoprotein has been found to be localized in the cortex of nodules, whose transcript level seems to be elevated upon increasing the ambient oxygen pressure (James et al., 1991). Therefore, multiple cell wall proteins may be involved in the oxygen regulation of nodule nitrogen fixation. Down-regulation of *Enod2* gene expression alone may not be sufficient to negatively influence nitrogenase activity.

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CHAPTER 6

CONCLUSIONS AND FUTURE PERSPECTIVES

Nodule Parenchyma Expression of the *SrEnod2* Gene

Nodulin genes are a group of plant nuclear genes induced in a spatio-temporal fashion during nodule development (Nap and Bisseling, 1990; Mylona et al., 1995). Their expression is believed to be mediated by the interaction between *cis*-acting DNA elements and *trans*-acting protein factors (de Bruijn and Schell, 1992). The dissection of the signal transduction pathways controlling nodulin gene expression is crucial for our understanding of how legume plants evolved to coordinate nodulin gene expression and for our future efforts to extend the beneficial Rhizobium-legume interaction to other agriculturally important crop plants such as the cereals.

The *Enod2* gene is one of the best characterized early nodulin genes, whose expression is localized in nodule parenchyma (van de Wiel et al., 1990). The *SrEnod2* gene isolated from the stem-nodulating legume *S. rostrata* is particularly interesting, because it is not only expressed during nodule development, but also in roots with the plant hormone cytokinin. Chapter 2 of this dissertation focuses on the developmental and tissue specific regulation of *SrEnod2* gene expression and shows that, unlike many other nodulin genes whose expression is controlled by their promoters (de Bruijn and Schell, 1992), the 3' untranslated region of the *SrEnod2* gene mediates nodule parenchyma-specific expression. In a separate study, the

same 3' UTR has been shown to be responsible for the cytokinin up-regulation of gene expression, which occurs post-transcriptionally in cytoplasm (D. Silver, Ph.D. dissertation research, Michigan State University, MI). It is plausible that the nodule developmental regulation and the cytokinin up-regulation of the *SrEnod2* gene expression involve the same mechanism. Regardless, these findings provide direct evidence for the first time that a 3' UTR confers cell-specific expression and that the cytokinin signal transduction pathway may be involved in nodule development.

There are several explanations for the observation that the 3' UTR mediates tissue-specific expression of the *SrEnod2* gene (see Chapter 2). One favored hypothesis is that the *SrEnod2* 3' UTR contains tissue-specific mRNA stabilization element(s) that are responsible for gene expression in the nodule parenchyma. Post-transcriptional regulation mechanisms clearly play important roles in the regulation of gene expression. Obviously, it is beneficial for a cell to use a post-transcriptional mechanism to maintain needed transcript levels.

Future experiments will focus on the isolation of *trans*-acting protein factors that interact with *cis*-elements located within the *SrEnod2* 3' UTR that may be responsible for tissue-specific expression. Chapter 3 describes the results of initial efforts to address this question. Using *in vitro* RNA-protein UV-cross linking assays, multiple proteins were found to interact

with *cis*-elements within the 3' UTR, but the functional significance of these findings will depend on the isolation of these RNA-binding proteins and the examination of their function *in vivo*. Techniques, such as the yeast three-hybrid system (SenGupta et al., 1996), and direct screening of cDNA expression libraries using riboprobes (Schumacher et al., 1995), may be useful for the isolation of RNA-binding proteins. One possible disadvantage of these techniques is that the selection procedure relies only on the physical, and not the biological properties of RNA. However, the tissue-specificity of putative RNA-binding proteins can be directly examined in nodules by northern analysis and by RNA *in-situ* hybridization. Thus, non-specific interacting proteins may be eliminated.

Other techniques to isolate *trans*-acting proteins that interact with the 3' UTR include genetic approaches using *Arabidopsis thaliana*. It has been shown that the *gus* reporter gene directed by the 5'*SrEnod2-gus*-3'*SrEnod2* construct (Figure 2-2A, Construct 1), is specifically induced by cytokinins in the roots of transgenic *Arabidopsis* plants (D. Silver, Ph.D. dissertation research). Therefore, *Arabidopsis* plants harboring both the 5'*SrEnod2-gus*-3'*SrEnod2* and the 5'*SrEnod2-codA*-3'*SrEnod2* constructs could be mutagenized and the M2 population screened for survival on medium containing 5-fluorocytosine (Perera et al., 1993; Stougaard, 1993). Mutant lines can be further examined for lack of the *gus* gene expression to make

sure that the mutations occurred in *trans*, and the corresponding genes could be isolated using positional cloning (Gibson and Somerville, 1992).

Ethylene Induction of the *LjEnod2* Gene

The finding that the *Enod2* gene from *L. japonicus* (*LjEnod2*) is inducible in roots by the plant hormone ethylene is interesting, because this suggests that ethylene may be involved in nodule development. Further support for this hypothesis could come from a direct comparison of the mechanisms of ethylene action and the developmental regulation of the *LjEnod2* gene. Ethylene responsible elements (EREs) have been identified in the promoter regions of several ethylene-inducible genes (Ohme-Takagi and Shinshi, 1995; Shinshi et al., 1995; Sessa et al., 1995; Nicholass et al., 1995; and Sato et al., 1996). It is quite possible that the promoter region of the *LjEnod2* gene contains such EREs. However, this raises several interesting questions about the finding that *Enod2* genes from different legumes are regulated differently. Are there any links between cytokinin and ethylene actions in *Enod2* gene expression? How are these different signaling pathways incorporated into the nodule organogenesis process?.

The Role of Enod2 Protein in Nodule Development

The Enod2 protein has been speculated to be involved in formation of an O_2 diffusion barrier in nodules (Nap and Bisseling, 1990; van de Wiel et al., 1990). Transgenic *L. corniculatus* plants transformed with antisense *Enod2* constructs form small nodules as compared to wild type plants, suggesting an alteration in nodule morphogenesis. Furthermore, the O_2 profile of several of these transgenic antisense plants appears to be different from wild type plants, suggesting an alteration in resistance to O_2 diffusion. However, these analyses have been complicated by the observation that the endogenous *Enod2* transcript levels were altered, but not abolished. Generation of a null mutant line might eventually prove the hypothesis that Enod2 protein contributes to the formation of the O_2 diffusion barrier in nodules. The model legume plant, *L. japonicus*, which contains a single *Enod2* gene, may prove to be the best system to study *Enod2* function. Additionally, alternative vehicles to express antisense RNAs, such as rRNA which is successfully used in *Tetrahymena thermophila* (Sweeney et al., 1996), may be exploited for a high level of expression of antisense *Enod2* transcripts and for efficient gene silencing. These will be the future projects in our lab.

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